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(54) Title: BMP-9 COMPOSITIONS (57) Abstract <p>Purified BMP-9 proteins and processes for producing them are disclosed. The proteins may be used in the treatment of bone and cartilage defects and in wound healing and related tissue repair, and in hepatic growth and function.</p>		

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BMP-9 COMPOSITIONS

The present invention relates to a novel family of purified proteins designated BMP-9 proteins and processes for obtaining them. These proteins may be used to induce bone and/or cartilage formation, in wound healing and tissue repair, and in hepatic growth and function.

The murine BMP-9 DNA sequence (SEQ ID NO:1) and amino acid sequence (SEQ ID NO:2) are set forth in Figure 1. Human BMP-9 sequence is set forth in Figure 3 (SEQ ID NO:8 and SEQ ID NO:9). It is contemplated that BMP-9 proteins are capable of inducing the formation of cartilage and/or bone. BMP-9 proteins may be further characterized by the ability to demonstrate cartilage and/or bone formation activity in the rat bone formation assay described below.

Murine BMP-9 is characterized by comprising amino acid #319 to #428 of Figure 1 (SEQ ID NO:2 amino acids #1-110). Murine BMP-9 may be produced by culturing a cell transformed with a DNA sequence comprising nucleotide #610 to nucleotide #1893 as shown in Figure 1 (SEQ ID NO:1) and recovering and purifying from the culture medium a protein characterized by the amino acid sequence comprising amino acid #319 to #428 as shown in Figure 1 (SEQ ID NO:2) substantially free from other proteinaceous materials with which it is co-produced.

Human BMP-9 is expected to be homologous to murine BMP-9 and is characterized by comprising amino acid #1 (Ser, Ala, Gly) to #110 of Figure 3 (SEQ ID NO:9) (Arg). The invention includes methods for obtaining the DNA sequences encoding human BMP-9. This method entails utilizing the murine BMP-9 nucleotide sequence or portions thereof to design probes to screen libraries for the human gene or fragments thereof using standard techniques. Human BMP-9 may be produced by culturing a cell transformed with the BMP-9 DNA sequence and recovering and purifying BMP-9 from the culture medium. The expressed protein is isolated, recovered, and purified from the culture medium.

The purified expressed protein is substantially free from other proteinaceous materials with which it is co-produced, as well as from other contaminants. The recovered purified protein is contemplated to exhibit cartilage and/or bone formation activity.

5 The proteins of the invention may be further characterized by the ability to demonstrate cartilage and/or bone formation activity in the rat bone formation assay described below.

Human BMP-9 may be produced by culturing a cell transformed with a DNA sequence comprising nucleotide #124 to #453 as shown
10 in SEQ ID NO:8 and recovering and purifying from the culture medium a protein characterized by the amino acid sequence of SEQ ID NO:9 from amino acid #1 to amino acid #110 substantially free from other proteinaceous materials with which it is co-produced.

Another aspect of the invention provides pharmaceutical
15 compositions containing a therapeutically effective amount of a BMP-9 protein in a pharmaceutically acceptable vehicle or carrier. BMP-9 compositions of the invention may be used in the formation of cartilage. These compositions may further be utilized for the formation of bone. BMP-9 compositions may also
20 be used for wound healing and tissue repair. Compositions of the invention may further include at least one other therapeutically useful agent such as the BMP proteins BMP-1, BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, and BMP-7 disclosed for instance in PCT Publication Nos. WO88/00205, WO89/10409, and WO90/11366, and BMP-8, disclosed
25 in U.S. Application Serial No. 07/641,204 filed January 15, 1991, Serial No. 07/525,357 filed May 16, 1990, and Serial No. 07/800,364 filed November 20, 1991.

The compositions of the invention may comprise, in addition to a BMP-9 protein, other therapeutically useful agents including
30 growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor (TGF- α and TGF- β), and insulin-like growth factor (IGF). The compositions may also include an appropriate matrix for instance, for supporting the composition and providing a surface for bone and/or cartilage
35 growth. The matrix may provide slow release of the osteoinductive protein and/or the appropriate environment for presentation thereof.

The BMP-9 compositions may be employed in methods for treating a number of bone and/or cartilage defects, periodontal disease and various types of wounds. These methods, according to the invention, entail administering to a patient needing such bone and/or cartilage formation wound healing or tissue repair, an effective amount of a BMP-9 protein. These methods may also entail the administration of a protein of the invention in conjunction with at least one of the novel BMP proteins disclosed in the co-owned applications described above. In addition, these methods may also include the administration of a BMP-9 protein with other growth factors including EGF, FGF, TGF- α , TGF- β , and IGF.

Still a further aspect of the invention are DNA sequences coding for expression of a BMP-9 protein. Such sequences include the sequence of nucleotides in a 5' to 3' direction illustrated in Figure 1 (SEQ ID NO:1) and Figure 3 (SEQ ID NO:8) or DNA sequences which hybridize under stringent conditions with the DNA sequences of Figure 1 or 3 and encode a protein having the ability to induce the formation of cartilage and/or bone. Finally, allelic or other variations of the sequences of Figure 1 or 3, whether such nucleotide changes result in changes in the peptide sequence or not, are also included in the present invention.

A further aspect of the invention includes vectors comprising a DNA sequence as described above in operative association with an expression control sequence therefor. These vectors may be employed in a novel process for producing a BMP-9 protein of the invention in which a cell line transformed with a DNA sequence encoding a BMP-9 protein in operative association with an expression control sequence therefor, is cultured in a suitable culture medium and a BMP-9 protein is recovered and purified therefrom. This process may employ a number of known cells both prokaryotic and eukaryotic as host cells for expression of the polypeptide.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description and preferred embodiments thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 comprises DNA sequence and derived amino acid sequence of murine BMP-9 from clone ML14a further described below.

5 FIG. 2 comprises DNA sequence and derived amino acid sequence of human BMP-4 from lambda U2OS-3 ATCC #40342.

FIG. 3 comprises DNA sequence and derived amino acid sequence of human BMP-9 from λ FIX/H6111 ATCC #75252.

FIG. 4 sets forth articular cartilage assay sulfate incorporation results.

10 FIG. 5 sets forth results of specific BMP-9 binding to HepG2 cells.

FIG. 6 sets forth results of stimulation of HepG2 cell proliferation by BMP-9.

15 FIG. 7 sets forth the results of stimulation of primary rat hepatocytes by BMP-9.

DETAILED DESCRIPTION OF THE INVENTION

The murine BMP-9 nucleotide sequence (SEQ ID NO:1) and encoded amino acid sequence (SEQ ID NO:2) are depicted in Figure 1. Purified murine BMP-9 proteins of the present invention are
20 produced by culturing a host cell transformed with a DNA sequence comprising the DNA coding sequence of Figure 1 (SEQ ID NO:1) from nucleotide #610 to nucleotide #1893 and recovering and purifying from the culture medium a protein which contains the amino acid sequence or a substantially homologous sequence as represented
25 by amino acid #319 to #428 of Figure 1 (SEQ ID NO:2). The BMP-9 proteins recovered from the culture medium are purified by isolating them from other proteinaceous materials from which they are co-produced and from other contaminants present.

Human BMP-9 nucleotide and amino acid sequence is depicted
30 in SEQ ID No: 8 and 9. Mature human BMP-9 is expected to comprise amino acid #1 (Ser, Ala, Gly) to #110 (Arg).

Human BMP-9 may be produced by culturing a cell transformed with a DNA sequence comprising nucleotide #124 to #453 as shown in SEQ ID NO:8 and recovering and purifying from the culture
35 medium a protein characterized by the amino acid sequence of SEQ ID NO:9 from amino acid #1 to amino acid #110 substantially free

from other proteinaceous materials with which it is co-produced.

BMP-9 proteins may be characterized by the ability to induce the formation of cartilage. BMP-9 proteins may be further characterized by the ability to induce the formation of bone. BMP-9 proteins may be further characterized by the ability to demonstrate cartilage and/or bone formation activity in the rat bone formation assay described below.

The BMP-9 proteins provided herein also include factors encoded by the sequences similar to those of Figure 1 and 3 (SEQ ID NOS:1 and 8), but into which modifications are naturally provided (e.g., allelic variations in the nucleotide sequence which may result in amino acid changes in the polypeptide) or deliberately engineered. For example, synthetic polypeptides may wholly or partially duplicate continuous sequences of the amino acid residues of Figure 1 of Figure 3 (SEQ ID NOS:2 and 9). These sequences, by virtue of sharing primary, secondary, or tertiary structural and conformational characteristics with bone growth factor polypeptides of Figure 1 and Figure 3 may possess bone growth factor biological properties in common therewith. Thus, they may be employed as biologically active substitutes for naturally-occurring BMP-9 and other BMP-9 polypeptides in therapeutic processes.

Other specific mutations of the sequences of BMP-9 proteins described herein involve modifications of glycosylation sites. These modifications may involve O-linked or N-linked glycosylation sites. For instance, the absence of glycosylation or only partial glycosylation results from amino acid substitution or deletion at asparagine-linked glycosylation recognition sites. The asparagine-linked glycosylation recognition sites comprise tripeptide sequences which are specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either asparagine-X-threonine or asparagine-X-serine, where X is usually any amino acid. A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) results in non-glycosylation at the modified

tripeptide sequence.

The present invention also encompasses the novel DNA sequences, free of association with DNA sequences encoding other proteinaceous materials, and coding on expression for BMP-9 proteins. These DNA sequences include those depicted in Figure 1 or Figure 3 (SEQ ID NOS:1 and 8) in a 5' to 3' direction and those sequences which hybridize thereto under stringent hybridization conditions [see, T. Maniatis et al., Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory (1982), pages 387 to 389] and encode a protein having cartilage and/or bone inducing activity.

Similarly, DNA sequences which code for BMP-9 proteins coded for by the sequences of Figure 1 or Figure 3, but which differ in codon sequence due to the degeneracies of the genetic code or allelic variations (naturally-occurring base changes in the species population which may or may not result in an amino acid change) also encode the novel factors described herein. Variations in the DNA sequences of Figure 1 or Figure 3 (SEQ ID NOS:1 and 8) which are caused by point mutations or by induced modifications (including insertion, deletion, and substitution) to enhance the activity, half-life or production of the polypeptides encoded are also encompassed in the invention.

Another aspect of the present invention provides a novel method for producing BMP-9 proteins. The method of the present invention involves culturing a suitable cell line, which has been transformed with a DNA sequence encoding a BMP-9 protein of the invention, under the control of known regulatory sequences. The transformed host cells are cultured and the BMP-9 proteins recovered and purified from the culture medium. The purified proteins are substantially free from other proteins with which they are co-produced as well as from other contaminants.

Suitable cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO). The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening, product production and purification are known in the art. See e.g., Gething and Sambrook, Nature 293:620-625 (1981), or alternatively, Kaufman et al., Mol. Cell.

Biol. 5(7):1750-1759 (1985) or Howley et al., U.S. Patent No. 4,419,446. Another suitable mammalian cell line, which is described in the accompanying examples, is the monkey COS-1 cell line. The mammalian cell CV-1 may also be suitable.

5 Bacterial cells may also be suitable hosts. For example, the various strains of E. coli (e.g., HB101, MC1061) are well-known as host cells in the field of biotechnology. Various strains of B. subtilis, Pseudomonas, other bacilli and the like may also be employed in this method.

10 Many strains of yeast cells known to those skilled in the art may also be available as host cells for expression of the polypeptides of the present invention. Additionally, where desired, insect cells may be utilized as host cells in the method of the present invention. See e.g., Miller et al., Genetic
15 Engineering 8:277-298 (Plenum Press 1986) and references cited therein.

Another aspect of the present invention provides vectors for use in the method of expression of these novel BMP-9 polypeptides. Preferably the vectors contain the full novel DNA
20 sequences described above which encode the novel factors of the invention. Additionally the vectors also contain appropriate expression control sequences permitting expression of the BMP-9 protein sequences. Alternatively, vectors incorporating modified sequences as described above are also embodiments of the present
25 invention. The vectors may be employed in the method of transforming cell lines and contain selected regulatory sequences in operative association with the DNA coding sequences of the invention which are capable of directing the replication and expression thereof in selected host cells. Regulatory sequences
30 for such vectors are known to those skilled in the art and may be selected depending upon the host cells. Such selection is routine and does not form part of the present invention.

A protein of the present invention, which induces cartilage and/or bone formation in circumstances where bone is not normally
35 formed, has application in the healing of bone fractures and cartilage defects in humans and other animals. Such a preparation employing a BMP-9 protein may have prophylactic use

in closed as well as open fracture reduction and also in the improved fixation of artificial joints. De novo bone formation induced by an osteogenic agent contributes to the repair of congenital, trauma induced, or oncologic resection induced craniofacial defects, and also is useful in cosmetic plastic surgery. A BMP-9 protein may be used in the treatment of periodontal disease, and in other tooth repair processes. Such agents may provide an environment to attract bone-forming cells, stimulate growth of bone-forming cells or induce differentiation of progenitors of bone-forming cells. BMP-9 polypeptides of the invention may also be useful in the treatment of osteoporosis. BMP-9 may be used in cartilage defect repair and prevention/reversal of osteoarthritis. A variety of osteogenic, cartilage-inducing and bone inducing factors have been described. See e.g., European Patent Application Nos. 148,155 and 169,016 for discussions thereof.

The proteins of the invention may also be used in wound healing and related tissue repair. The types of wounds include, but are not limited to burns, incisions and ulcers. (See e.g., PCT Publication No. WO84/01106 for discussion of wound healing and related tissue repair).

It is further contemplated that proteins of the invention may increase neuronal survival and therefore be useful in transplantation and treatment of conditions exhibiting a decrease in neuronal survival.

BMP-9 proteins of the invention may also be useful in hepatic growth and function including repair and regeneration of liver cells. BMP-9 may therefore be used for instance in treatment of conditions exhibiting degeneration of the liver.

A further aspect of the invention is a therapeutic method and composition for repairing fractures and other conditions related to cartilage and/or bone defects or periodontal diseases. The invention further comprises therapeutic methods and compositions for wound healing and tissue repair. Such compositions comprise a therapeutically effective amount of at least one of the BMP-9 proteins of the invention in admixture with a pharmaceutically acceptable vehicle, carrier or matrix.

It is expected that the proteins of the invention may act in concert with or perhaps synergistically with other related proteins and growth factors. Further therapeutic methods and compositions of the invention therefore comprise a therapeutic amount of at least one BMP-9 protein of the invention with a therapeutic amount of at least one of the other BMP proteins disclosed in co-owned applications described above. Such combinations may comprise separate molecules of the BMP proteins or heteromolecules comprised of different BMP moieties. For example, a method and composition of the invention may comprise a disulfide linked dimer comprising a BMP-9 protein subunit and a subunit from one of the "BMP" proteins described above. A further embodiment may comprise a heterodimer of BMP-9 moieties. Further, BMP-9 proteins may be combined with other agents beneficial to the treatment of the bone and/or cartilage defect, wound, or tissue in question. These agents include various growth factors such as epidermal growth factor (EGF), platelet derived growth factor (PDGF), transforming growth factors (TGF- α and TGF- β), and insulin-like growth factor (IGF).

The preparation and formulation of such physiologically acceptable protein compositions, having due regard to pH, isotonicity, stability and the like, is within the skill of the art. The therapeutic compositions are also presently valuable for veterinary applications due to the lack of species specificity in BMP proteins. Particularly domestic animals and thoroughbred horses in addition to humans are desired patients for such treatment with BMP-9 of the present invention.

The therapeutic method includes administering the composition topically, systemically, or locally as an implant or device. When administered, the therapeutic composition for use in this invention is, of course, in a pyrogen-free, physiologically acceptable form. Further, the composition may desirably be encapsulated or injected in a viscous form for delivery to the site of bone, cartilage or tissue damage. Topical administration may be suitable for wound healing and tissue repair. Therapeutically useful agents other than the BMP-9 proteins which may also optionally be included in the

composition as described above, may alternatively or additionally, be administered simultaneously or sequentially with the BMP composition in the methods of the invention. Preferably for bone and/or cartilage formation, the composition would include a matrix capable of delivering BMP-9 or other BMP proteins to the site of bone and/or cartilage damage, providing a structure for the developing bone and cartilage and optimally capable of being resorbed into the body. The matrix may provide slow release of BMP-9 and/or the appropriate environment for presentation thereof. Such matrices may be formed of materials presently in use for other implanted medical applications.

The choice of matrix material is based on biocompatibility, biodegradability, mechanical properties, cosmetic appearance and interface properties. The particular application of the BMP-9 compositions will define the appropriate formulation. Potential matrices for the compositions may be biodegradable and chemically defined calcium sulfate, tricalciumphosphate, hydroxyapatite, polylactic acid and polyanhydrides. Other potential materials are biodegradable and biologically well defined, such as bone or dermal collagen. Further matrices are comprised of pure proteins or extracellular matrix components. Other potential matrices are nonbiodegradable and chemically defined, such as sintered hydroxyapatite, bioglass, aluminates, or other ceramics. Matrices may be comprised of combinations of any of the above mentioned types of material, such as polylactic acid and hydroxyapatite or collagen and tricalciumphosphate. The bioceramics may be altered in composition, such as in calcium-aluminate-phosphate and processing to alter pore size, particle size, particle shape, and biodegradability.

The dosage regimen will be determined by the attending physician considering various factors which modify the action of the BMP-9 protein, e.g., amount of bone weight desired to be formed, the site of bone damage, the condition of the damaged bone, the size of a wound, type of damaged tissue, the patient's age, sex, and diet, the severity of any infection, time of administration and other clinical factors. The dosage may vary with the type of matrix used in the reconstitution and the types

of BMP proteins in the composition. The addition of other known growth factors, such as IGF I (insulin like growth factor I), to the final composition, may also effect the dosage. Progress can be monitored by periodic assessment of bone growth and/or repair, for example, x-rays, histomorphometric determinations and tetracycline labeling.

The following examples illustrate practice of the present invention in recovering and characterizing murine BMP-9 protein and employing it to recover the human and other BMP-9 proteins, obtaining the human proteins and expressing the proteins via recombinant techniques.

EXAMPLE I

MURINE BMP-9

750,000 recombinants of a mouse liver cDNA library made in the vector lambdaZAP (Stratagene/Catalog #935302) are plated and duplicate nitrocellulose replicas made. A fragment of human BMP-4 DNA corresponding to nucleotides 1330-1627 of Figure 2 (SEQ ID NO:3) (the human BMP-4 sequence) is ³²P-labeled by the random priming procedure of Feinberg et al., Anal. Biochem. 132:6-13 (1983) and hybridized to both sets of filters in SHB at 60°C for 2 to 3 days. Both sets of filters are washed under reduced stringency conditions (4X SSC, 0.1% SDS at 60°C). Many duplicate hybridizing recombinants of various intensities (approximately 92) are noted. 50 of the strongest hybridizing recombinant bacteriophage are plaque purified and their inserts are transferred to the plasmid Bluescript SK (+/-) according to the in vivo excision protocol described by the manufacturer (Stratagene). DNA sequence analysis of several recombinants indicate that they encode a protein homologous to other BMP proteins and other proteins in the TGF- β family. The DNA sequence and derived amino acid sequence of one recombinant, designated ML14a, is set forth in Figure 1. (SEQ ID NO:1)

The nucleotide sequence of clone ML14a contains an open reading frame of 1284 bp, encoding a BMP-9 protein of 428 amino acids. The encoded 428 amino acid BMP-9 protein is contemplated to be the primary translation product as the coding sequence is

preceded by 609 bp of 5' untranslated sequence with stop codons in all three reading frames. The 428 amino acid sequence predicts a BMP-9 protein with a molecular weight of 48,000 daltons.

5 Based on knowledge of other BMP proteins and other proteins within the TGF- β family, it is predicted that the precursor polypeptide would be cleaved at the multibasic sequence ARG-ARG-LYS-ARG in agreement with a proposed consensus proteolytic processing sequence of ARG-X-X-ARG. Cleavage of the BMP-9 precursor polypeptide at this location would generate a 110 amino acid mature peptide beginning with the amino acid SER at position 10 #319. The processing of BMP-9 into the mature form is expected to involve dimerization and removal of the N-terminal region in a manner analogous to the processing of the related protein TGF- β 15 [L. E. Gentry et al., Mol. & Cell. Biol. 8:4162 (1988); R. Derynck et al., Nature 316:701 (1985)].

It is contemplated therefore that the mature active species of murine BMP-9 comprises a homodimer of 2 polypeptide subunits, each subunit comprising amino acids #319-#428 with a predicted 20 molecular weight of approximately 12,000 daltons. Further active species are contemplated comprising amino acids #326 - #428 thereby including the first conserved cysteine residue. As with other members of the BMP and TGF- β family of proteins, the carboxy-terminal region of the BMP-9 protein exhibits greater 25 sequence conservation than the more amino-terminal portion. The percent amino acid identity of the murine BMP-9 protein in the cysteine-rich C-terminal domain (amino acids #326 - #428) to the corresponding region of other human BMP proteins and other proteins within the TGF- β family is as follows: BMP-2, 53%; BMP- 30 3, 43%; BMP-4, 53%; BMP-5, 55%; BMP-6, 55%; BMP-7, 53%; Vgl, 50%; GDF-1, 43%; TGF- β 1, 32%; TGF- β 2, 34%; TGF- β 3, 34%; inhibin β (B), 34%; and inhibin β (A), 42%.

EXAMPLE II

HUMAN BMP-9

35 Murine and human osteoinductive factor genes are presumed to be significantly homologous, therefore the murine coding

sequence or a portion thereof is used as a probe to screen a human genomic library or as a probe to identify a human cell line or tissue which synthesizes the analogous human cartilage and/or bone protein. A human genomic library (Toole et al., supra) may be screened with such a probe, and presumptive positives isolated and DNA sequence obtained. Evidence that this recombinant encodes a portion of the human BMP-9 relies on the murine/human protein and gene structure homologies.

Once a recombinant bacteriophage containing DNA encoding portion of the human cartilage and/or bone inductive factor molecule is obtained, the human coding sequence can be used as a probe to identify a human cell line or tissue which synthesizes BMP-9. Alternatively, the murine coding sequence can be used as a probe to identify such human cell line or tissue. Briefly described, RNA is extracted from a selected cell or tissue source and either electrophoresed on a formaldehyde agarose gel and transferred to nitrocellulose, or reacted with formaldehyde and spotted on nitrocellulose directly. The nitrocellulose is then hybridized to a probe derived from a coding sequence of the murine or human BMP-9. mRNA is selected by oligo (dT) cellulose chromatography and cDNA is synthesized and cloned in lambda gt10 or lambda ZAP by established techniques (Toole et al., supra).

Additional methods known to those skilled in the art may be used to isolate the human and other species' BMP-9 proteins of the invention.

A. ISOLATION OF HUMAN BMP-9 DNA

One million recombinants of a human genomic library constructed in the vector λ FIX (Stratagene catalog # 944201) are plated and duplicate nitrocellulose replicas made. Two oligonucleotide probes designed on the basis of nucleotides #1665-#1704 and #1837-#1876 of the sequence set forth in Figure 1 (SEQ ID NO:1) are synthesized on an automated DNA synthesizer. The sequence of these two oligonucleotides is indicated below:

#1: CTATGAGTGTAAGGGGGTTGCTTCTTCCCATTTGGCTGAT

#2: GTGCCAACCTCAAGTACCACTATGAGGGGATGAGTGTGG

These two oligonucleotide probes are radioactively labeled with

$\gamma^{32}\text{P}$ -ATP and each is hybridized to one set of the duplicate nitrocellulose replicas in SHB at 65°C and washed with 1X SSC, 0.1% SDS at 65°C. Three recombinants which hybridize to both oligonucleotide probes are noted. All three positively hybridizing recombinants are plaque purified, bacteriophage plate stocks are prepared and bacteriophage DNA is isolated from each. The oligonucleotide hybridizing regions of one of these recombinants, designated HG111, is localized to a 1.2 kb Pst I/Xba I fragment. This fragment is subcloned into a plasmid vector (pGEM-3) and DNA sequence analysis is performed. HG111 was deposited with the American Type Culture Collection ATCC, 12301 Parklawn Drive, Rockville, Maryland USA (hereinafter the "ATCC") on June 16, 1992 under the requirements of the Budapest Treaty and designated as ATCC #75252. This subclone is designated pGEM-111. A portion of the DNA sequence of clone pGEM-111 is set forth in Figure 3 (SEQ ID NO:8/HUMAN BMP-9 SEQUENCE). This sequence encodes the entire mature region of human BMP-9 and a portion of the propeptide. It should be noted that this sequence consists of preliminary data. Particularly, the propeptide region is subject to further analysis and characterization. For example, nucleotides #1 through #3 (TGA) encode a translational stop which may be incorrect due to the preliminary nature of the sequence. It is predicted that additional sequences present in both pGEM-111 (the 1.2 kb PstI/XbaI fragment of HG111 subcloned into pGEM) and HG111 encode additional amino acids of the human BMP-9 propeptide region. Based on knowledge of other BMPs and other proteins within the TGF- β family, it is predicted that the precursor polypeptide would be cleaved at the multibasic sequence ARG-ARG-LYS-ARG (amino acids # -4 through # -1 of SEQ ID NO:9) in agreement with a proposed consensus proteolytic processing sequence ARG-X-X-ARG. Cleavage of the human BMP-9 precursor polypeptide at this location would generate a 110 amino acid mature peptide beginning with the amino acid SER at position #1 of SEQ ID NO:9 (encoded by nucleotides #124 through #126 of SEQ ID NO:8). The processing of human BMP-9 into the mature form is expected to involve dimerization and removal of the N-terminal region in a manner

analogous to the processing of the related protein TGF- β [L.E. Gentry et al., Mol. & Cell. Biol. 8:4162 (1988); R. Derynck et al., Nature 316:701 (1985)].

5 It is contemplated therefore that the mature active species of human BMP-9 comprises a homodimer of two polypeptide subunits, each subunit comprising amino acids #1 through #110 of SEQ ID NO:9, with a predicted molecular weight of 12,000 daltons. Further active species are contemplated comprising amino acids
10 #8 through #110 thereby including the first conserved cysteine residue. As with other members of the BMP and TGF- β family of proteins, the carboxy-terminal portion of the human BMP-9 sequence exhibits greater sequence conservation than the amino-terminal portion. The percent amino acid identity of the human BMP-9 protein in the cysteine-rich C-terminal domain (amino acids
15 #8 through #110) to the corresponding region of other human BMP proteins and other proteins within the TGF- β family is as follows: BMP-2, 52%; BMP-3, 40%; BMP-4, 52%; BMP-5, 55%; BMP-6, 55%; BMP-7, 53%; murine BMP-9, 97%; Vg1, 50%; GDF-1, 44%; TGF- β 1, 32%; TGF- β 2, 32%; TGF- β 3, 32%; inhibin β (B), 35%; and inhibin
20 β (A), 41%. BMP-9 exhibits 80% homology to chick Dorsalin-1, a BMP-like protein cloned from embryonic chick.

EXAMPLE III

ROSEN MODIFIED SAMPATH-REDDI ASSAY

25 A modified version of the rat bone formation assay described in Sampath and Reddi, Proc. Natl. Acad. Sci. USA 80:6591-6595 (1983) is used to evaluate bone and/or cartilage activity of the BMP proteins. This modified assay is herein called the Rosen-modified Sampath-Reddi assay. The ethanol precipitation step of the Sampath-Reddi procedure is replaced by dialyzing (if the
30 composition is a solution) or diafiltering (if the composition is a suspension) the fraction to be assayed against water. The solution or suspension is then redissolved in 0.1 % TFA, and the resulting solution added to 20mg of rat matrix. A mock rat matrix sample not treated with the protein serves as a control.
35 This material is frozen and lyophilized and the resulting powder enclosed in #5 gelatin capsules. The capsules are implanted

subcutaneously in the abdominal thoracic area of 21 - 49 day old male Long Evans rats. The implants are removed after 7 - 14 days. Half of each implant is used for alkaline phosphatase analysis [See, A. H. Reddi et al., Proc. Natl Acad Sci. 69:1601 (1972)].

5 The other half of each implant is fixed and processed for histological analysis. 11m glycolmethacrylate sections are stained with Von Kossa and acid fuchsin to score the amount of induced bone and cartilage formation present in each implant. 10 The terms +1 through +5 represent the area of each histological section of an implant occupied by new bone and/or cartilage cells and matrix. A score of +5 indicates that greater than 50% of the implant is new bone and/or cartilage produced as a direct result of protein in the implant. A score of +4, +3, +2 and 15 +1 would indicate that greater than 40%, 30%, 20% and 10% respectively of the implant contains new cartilage and/or bone. In a modified scoring method, three non-adjacent sections are evaluated from each implant and averaged. "+/-" indicates tentative identification of cartilage or bone; "+1" indicates 20 >10% of each section being new cartilage or bone; "+2", >25%; "+3", >50%; "+4", ~75%; "+5", >80%. A "-" indicates that the implant is not recovered.

It is contemplated that the dose response nature of the BMP-9 containing samples of the matrix samples will demonstrate that 25 the amount of bone and/or cartilage formed increases with the amount of BMP-9 in the sample. It is contemplated that the control samples will not result in any bone and/or cartilage formation.

30 As with other cartilage and/or bone inductive proteins such as the above-mentioned "BMP" proteins, the bone and/or cartilage formed is expected to be physically confined to the space occupied by the matrix. Samples are also analyzed by SDS gel electrophoresis and isoelectric focusing followed by autoradiography. The activity is correlated with the protein 35 bands and pI. To estimate the purity of the protein in a particular fraction an extinction coefficient of 1 OD/mg-cm is used as an estimate for protein and the protein is run on SDS

PAGE followed by silver staining or radioiodination and autoradiography.

EXAMPLE IV

EXPRESSION OF BMP-9

5 In order to produce murine, human or other mammalian BMP-9 proteins, the DNA encoding it is transferred into an appropriate expression vector and introduced into mammalian cells or other preferred eukaryotic or prokaryotic hosts by conventional genetic engineering techniques. The preferred
10 expression system for biologically active recombinant human BMP-9 is contemplated to be stably transformed mammalian cells.

One skilled in the art can construct mammalian expression vectors by employing the sequence of Figure 1 (SEQ ID NO:1) or Figure 3 (SEQ ID NO:8), or other DNA sequences encoding BMP-9
15 proteins or other modified sequences and known vectors, such as pCD [Okayama et al., Mol. Cell Biol. 2:161-170 (1982)], pJL3, pJL4 [Gough et al., EMBO J. 4:645-653 (1985)] and pMT2 CXM.

The mammalian expression vector pMT2 CXM is a derivative of p91023 (b) (Wong et al., Science 228:810-815 (1985)) differing
20 from the latter in that it contains the ampicillin resistance gene in place of the tetracycline resistance gene and further contains a XhoI site for insertion of cDNA clones. The functional elements of pMT2 CXM have been described (Kaufman, R.J., Proc. Natl. Acad. Sci. USA 82:689-693 (1985)) and include
25 the adenovirus VA genes, the SV40 origin of replication including the 72 bp enhancer, the adenovirus major late promoter including a 5' splice site and the majority of the adenovirus tripartite leader sequence present on adenovirus late mRNAs, a 3' splice acceptor site, a DHFR insert, the SV40 early polyadenylation site
30 (SV40), and pBR322 sequences needed for propagation in E. coli.

Plasmid pMT2 CXM is obtained by EcoRI digestion of pMT2-VWF, which has been deposited with the American Type Culture Collection (ATCC), Rockville, MD (USA) under accession number ATCC #67122. EcoRI digestion excises the cDNA insert present in
35 pMT2-VWF, yielding pMT2 in linear form which can be ligated and used to transform E. coli HB 101 or DH-5 to ampicillin

resistance. Plasmid pMT2 DNA can be prepared by conventional methods. pMT2 CXM is then constructed using loopout/in mutagenesis [Morinaga et al., Biotechnology 84:636 (1984)]. This removes bases 1075 to 1145 relative to the Hind III site near the SV40 origin of replication and enhancer sequences of pMT2. In addition it inserts the following sequence:

5' PO-CATGGGCAGCTCGAG-3' (SEQ ID NO:5)

at nucleotide 1145. This sequence contains the recognition site for the restriction endonuclease Xho I. A derivative of pMT2CXM, termed pMT23, contains recognition sites for the restriction endonucleases PstI, Eco RI, SalI and XhoI. Plasmid pMT2 CXM and pMT23 DNA may be prepared by conventional methods.

pEMC2b1 derived from pMT21 may also be suitable in practice of the invention. pMT21 is derived from pMT2 which is derived from pMT2-VWF. As described above EcoRI digestion excises the cDNA insert present in pMT-VWF, yielding pMT2 in linear form which can be ligated and used to transform E. coli HB 101 or DH-5 to ampicillin resistance. Plasmid pMT2 DNA can be prepared by conventional methods.

pMT21 is derived from pMT2 through the following two modifications. First, 76 bp of the 5' untranslated region of the DHFR cDNA including a stretch of 19 G residues from G/C tailing for cDNA cloning is deleted. In this process, a XhoI site is inserted to obtain the following sequence immediately

upstream from DHFR: 5' -CTGCAGGCGAGCCTGAATTCCTCGAGCCATCATG-3'
PstI Eco RI XhoI
(SEQ ID NO:6)

Second, a unique ClaI site is introduced by digestion with EcoRV and XbaI, treatment with Klenow fragment of DNA polymerase I, and ligation to a ClaI linker (CATCGATG). This deletes a 250 bp segment from the adenovirus associated RNA (VAI) region but does not interfere with VAI RNA gene expression or function. pMT21 is digested with EcoRI and XhoI, and used to derive the vector pEMC2B1.

A portion of the EMCV leader is obtained from pMT2-ECAT1 [S.K. Jung et al., J. Virol 63:1651-1660 (1989)] by digestion with Eco RI and PstI, resulting in a 2752 bp fragment. This fragment is digested with TaqI yielding an Eco RI-TaqI fragment

of 508 bp which is purified by electrophoresis on low melting agarose gel. A 68 bp adapter and its complementary strand are synthesized with a 5' TaqI protruding end and a 3' XhoI protruding end which has the following sequence:

5 5'-CGAGGTTAAAAACGTCTAGGCCCCCGAACCACGGGGACGTGGTTTTCCTTT
TaqI

GA AAAACACGATTGC-3'
XhoI (SEQ ID NO:7)

10 This sequence matches the EMC virus leader sequence from nucleotide 763 to 827. It also changes the ATG at position 10 within the EMC virus leader to an ATT and is followed by a XhoI site. A three way ligation of the pMT21 Eco RI-XhoI fragment, the EMC virus EcoRI-TaqI fragment, and the 68 bp oligonucleotide
15 adapter TaqI-XhoI adapter resulting in the vector pEMC2 β 1.

This vector contains the SV40 origin of replication and enhancer, the adenovirus major late promoter, a cDNA copy of the majority of the adenovirus tripartite leader sequence, a small hybrid intervening sequence, an SV40 polyadenylation signal and
20 the adenovirus VA I gene, DHFR and β -lactamase markers and an EMC sequence, in appropriate relationships to direct the high level expression of the desired cDNA in mammalian cells.

The construction of vectors may involve modification of the BMP-9 DNA sequences. For instance, BMP-9 cDNA can be modified
25 by removing the non-coding nucleotides on the 5' and 3' ends of the coding region. The deleted non-coding nucleotides may or may not be replaced by other sequences known to be beneficial for expression. These vectors are transformed into appropriate host cells for expression of BMP-9 proteins. One skilled in the art
30 can manipulate the sequences of Figure 1 or Figure 3 (SEQ ID NOS:1 and 8) by eliminating or replacing the mammalian regulatory sequences flanking the coding sequence with bacterial sequences to create bacterial vectors for intracellular or extracellular expression by bacterial cells. For example, the coding sequences
35 could be further manipulated (e.g., ligated to other known linkers or modified by deleting non-coding sequences therefrom or altering nucleotides therein by other known techniques). The modified BMP-9 coding sequence could then be inserted into a

known bacterial vector using procedures such as described in T. Taniguchi et al., Proc. Natl Acad. Sci. USA 77:5230-5233 (1980). This exemplary bacterial vector could then be transformed into bacterial host cells and a BMP-9 protein expressed thereby. For
5 a strategy for producing extracellular expression of BMP-9 proteins in bacterial cells, see e.g., European Patent Application No. EPA 177,343.

Similar manipulations can be performed for the construction of an insect vector [See e.g., procedures described in published
10 European Patent Application No. 155,476] for expression in insect cells. A yeast vector could also be constructed employing yeast regulatory sequences for intracellular or extracellular expression of the factors of the present invention by yeast cells. [See e.g., procedures described in published PCT
15 Publication No. WO86/00639 and European Patent Application No. EPA 123,289].

A method for producing high levels of a BMP-9 protein of the invention in mammalian cells may involve the construction of cells containing multiple copies of the heterologous BMP-9 gene.
20 The heterologous gene is linked to an amplifiable marker, e.g., the dihydrofolate reductase (DHFR) gene for which cells containing increased gene copies can be selected for propagation in increasing concentrations of methotrexate (MTX) according to the procedures of Kaufman and Sharp, J. Mol. Biol. 159:601-629
25 (1982). This approach can be employed with a number of different cell types.

For example, a plasmid containing a DNA sequence for a BMP-9 of the invention in operative association with other plasmid sequences enabling expression thereof and the DHFR expression
30 plasmid pAdA26SV(A)3 [Kaufman and Sharp, Mol. Cell. Biol. 2:1304 (1982)] can be co-introduced into DHFR-deficient CHO cells, DUKX-BII, by various methods including calcium phosphate coprecipitation and transfection, electroporation or protoplast fusion. DHFR expressing transformants are selected for growth
35 in alpha media with dialyzed fetal calf serum, and subsequently selected for amplification by growth in increasing concentrations of MTX (e.g., sequential steps in 0.02, 0.2, 1.0 and 5uM MTX) as

described in Kaufman et al., Mol Cell Biol. 5:1750 (1983). Transformants are cloned, and biologically active BMP-9 expression is monitored by the Rosen-modified Sampath - Reddi rat bone formation assay described above in Example III. BMP-9 expression should increase with increasing levels of MTX resistance. BMP-9 polypeptides are characterized using standard techniques known in the art such as pulse labeling with [35S] methionine or cysteine and polyacrylamide gel electrophoresis. Similar procedures can be followed to produce other related BMP-9 proteins.

A. BMP-9 VECTOR CONSTRUCTION

In order to produce human BMP-9 proteins of the invention DNA sequences encoding the mature region of the human BMP-9 protein may be joined to DNA sequences encoding the propeptide region of the murine BMP-9 protein. This murine/human hybrid DNA sequence is inserted into an appropriate expression vector and introduced into mammalian cells or other preferred eukaryotic or prokaryotic hosts by conventional genetic engineering techniques. The construction of this murine/human BMP-9 containing expression plasmid is described below.

A derivative of the human BMP-9 sequence (SEQ ID NO:8) comprising the nucleotide sequence from nucleotide #105 to #470 is specifically amplified. The following oligonucleotides are utilized as primers to allow the amplification of nucleotides #105 to #470 of the human BMP-9 sequence (SEQ ID NO:8) from clone pGEM-111 described above.

#3 ATCGGGCCCCTTTTAGCCAGGCGGAAAAGGAG

#4 AGCGAATTCCTCCCGCAGGCAGATACTACCTG

This procedure generates the insertion of the nucleotide sequence ATCGGGCCCCT immediately preceding nucleotide #105 and the insertion of the nucleotide sequence GAATTCGCT immediately following nucleotide #470. The addition of these sequences results in the creation of an Apa I and EcoR I restriction endonuclease site at the respective ends of the specifically amplified DNA fragment. The resulting 374 bp Apa I/EcoR I fragment is subcloned into the plasmid vector pGEM-7Zf(+)

(Promega catalog# p2251) which has been digested with Apa I and EcoR I. The resulting clone is designated phBMP9mex-1.

The following oligonucleotides are designed on the basis of murine BMP-9 sequences (SEQ ID NO:1) and are modified to facilitate the construction of the murine/human expression plasmid referred to above:

#5

GATTCCGTCGACCACCATGTCCCCTGGGGCCTGGTCTAGATGGATACACAGCTGTGGGGCC

#6 CCACAGCTGTGTATCCATCTAGACCAGGCCCCAGGGGACATGGTGGTTCGACG

These oligonucleotides contain complimentary sequences which upon addition to each other facilitate the annealing (base pairing) of the two individual sequences, resulting in the formation of a double stranded synthetic DNA linker (designated LINK-1) in a manner indicated below:

15	1	5	10	20	30	40	50	60
	#5	GATTCCGTCGACCACCATGTCCCCTGGGGCCTGGTCTAGATGGATACACAGCTGTGGGGCC						
		GCAGCTGGTGGTACAGGGGACCCCGGACCAGATCTACCTATGTGTTCGACACC	#6					

This DNA linker (LINK-1) contains recognition sequences of restriction endonucleases needed to facilitate subsequent manipulations required to construct the murine/human expression plasmid, as well as sequences required for maximal expression of heterologous sequences in mammalian cell expression systems. More specifically (referring to the sequence numbering of oligonucleotide #5/LINK-1): nucleotides #1-#11 comprise recognition sequences for the restriction endonucleases BamH I and Sal I, nucleotides #11-#15 allow for maximal expression of heterologous sequences in mammalian cell expression systems, nucleotides #16-#31 correspond to nucleotides #610-#625 of the murine BMP-9 sequence (SEQ ID NO:1), nucleotides #32-#33 are inserted to facilitate efficient restriction digestion of two adjacent restriction endonuclease sites (EcoO109 I and Xba I), nucleotides #34-#60 correspond to nucleotides #1515-#1541 of the murine BMP-9 sequence (SEQ ID NO:1) except that nucleotide #58 of synthetic oligonucleotide #5 is a G rather than the A which appears at position #1539 of SEQ ID NO:1 (This nucleotide conversion results in the creation of an Apa I restriction endonuclease recognition sequence, without altering the amino

acid sequence it is intended to encode, to facilitate further manipulations of the murine/human hybrid expression plasmid. LINK-1 (the double stranded product of the annealing of oligonucleotides #5 and #6) is subcloned into the plasmid vector pGEM-7Zf(+) which has been digested with the restriction endonucleases Apa I and BamH I. This results in a plasmid in which the sequences normally present between the Apa I and BamH I sites of the pGEM-7Zf(+) plasmid polylinker are replaced with the sequences of LINK-1 described above. The resulting plasmid clone is designated pBMP-9link.

pBMP-9link is digested with the restriction endonucleases BamH I and Xba I resulting in the removal nucleotides #1-#34 of LINK-1 (refer to the numbering of oligo #5). Clone ML14a, which contains an insert comprising the sequence set forth in SEQ ID NO:1, is also digested with the restriction endonucleases BamH I and Xba I resulting in the removal of sequences comprising nucleotides #1-#1515 of SEQ ID NO:1 (murine BMP-9). This BamH I/Xba I fragment of mouse BMP-9 is isolated from the remainder of the ML14a plasmid clone and subcloned into the BamH I/Xba I sites generated by the removal of the synthetic linker sequences described above. The resulting clone is designated p302.

The p302 clone is digested with the restriction endonuclease EcoO109 I resulting in the excision of nucleotides corresponding to nucleotides #621-#1515 of the murine BMP-9 sequence (SEQ ID NO:1) and nucleotides #35-#59 of LINK-1 (refer to numbering of oligonucleotide #5). It should be noted that the Apa I restriction site created in LINK-1 by the A to G conversion described above is a subset of the recognition sequence of EcoO109 I, therefore digestion of p302 with EcoO109 I cleaves at the Apa I site as well as the naturally occurring murine EcoO109 I (location #619-#625 of SEQ ID NO:1) resulting in the excision of a 920 bp EcoO109 I/EcoO109 I (Apa I) fragment comprising the sequences described above. This 920 EcoO109 I/EcoO109 I (Apa I) fragment is isolated from the remainder of the p302 plasmid clone and subcloned into clone pBMP-9link which has been similarly digested with EcoO109 I. It should be noted that the nucleotides GG (#32-#33 of oligonucleotide #5) originally designed to

facilitate a more complete digestion of the two adjacent restriction sites EcoO109 I and Xba I of LINK-1, which is now a part of pBMP-9link (described above), results in the creation of Dcm methylation recognition sequence. The restriction nuclease EcoO109 I is sensitive to Dcm methylation and therefore cleavage of this sequence (nucleotides #25-#31 of oligonucleotide #5/LINK-1) by the restriction endonuclease EcoO109 I is prevented at this site. Therefore the plasmid clone pBMP-9link is cleaved at the Apa I site but not at the EcoO109 I site upon digestion with the restriction endonuclease EcoO109 I as described above, preventing the intended removal of the sequences between the EcoO109 I and Xba I site of LINK-1 (#32-#55 defined by the numbering of oligonucleotide #5). This results in the insertion of the 920 bp EcoO109 I/Apa I fragment at the EcoO109 I (Apa I) site of pBMP-9link. The resulting clone is designated p318.

Clone p318 is digested with the restriction endonucleases Sal I and Apa I, resulting in the excision of sequences comprising nucleotides #6-#56 of LINK-1 (refer to oligo #5 for location), nucleotides #621-#1515 of murine BMP-9 (SEQ ID NO:1), and nucleotides #35-#60 of LINK-1 (refer to oligo #5 for location). The resulting 972 bp Sal I/Apa I fragment described above is isolated from the remainder of the p318 plasmid clone and will be utilized in subsequent manipulations.

The clone phBMP9mex-1 (described above), which contains DNA sequences which encode the entire mature region and portions of the propeptide of the human BMP-9 protein, is digested with the restriction endonucleases Apa I and EcoR I. This results in the excision of a 374 bp fragment comprising nucleotides #105-#470 of the human BMP-9 sequence (SEQ ID NO:8) and the additional nucleotides of oligonucleotide primers #3 and #4 which contain the recognition sequences for the restriction endonucleases Apa I and EcoR I. This 374 bp Apa I/EcoR I fragment is combined with the 972 bp Sal I/Apa I fragment from p318 (isolation described above) and ligated to the mammalian cell expression plasmid pED6 (a derivative of pEMC2 β 1) which has been digested with Sal I and EcoR I. The resulting clone is designated p324.

The clone ML14a (murine BMP-9) is digested with EcoO109 I

and Xba I to generate a fragment comprising nucleotides #621-#1515 of SEQ ID NO:1.

5 The following oligonucleotides are synthesized on an automated DNA synthesizer and combined such that their complimentary sequences can base pair (anneal) with each other to generate a double stranded synthetic DNA linker designated LINK-2:

#7 TCGACCACCATGTCCCCTGG

#8 GCCCCAGGGGACATGGTGG

10 This double stranded synthetic DNA linker (LINK-2) anneals in such a way that it generates single stranded ends which are compatible to DNA fragments digested with Sal I (one end) or EcoO109 I (the other end) as indicated below:

15 #7 TCGACCACCATGTCCCCTGG
GGTGGTACAGGGGACCCCG #8

This LINK-2 synthetic DNA linker is ligated to the 895 bp EcoO109 I/Xba I fragment comprising nucleotides #621-#1515 of murine BMP-9 (SEQ ID NO:1) described above. This results in a 915 bp Sal I/Xba I fragment.

20 The clone p324 is digested with Sal I/Xba I to remove sequences comprising nucleotides #6-#56 of LINK-1 (refer to oligo #5 for location) and nucleotides #621-#1515 of murine BMP-9 (SEQ ID NO:1). The sequences comprising nucleotides #35-#60 of LINK-1 (refer to oligo #5 for location) and the sequences comprising the
25 374 bp Apa I/EcoR I fragment (human BMP-9 sequences) derived from phBMP9mex-1 remain attached to the pED6 backbone. The 915 bp Sal I/Xba I fragment comprising LINK-2 sequences and nucleotides #621-#1515 of murine BMP-9 (SEQ ID NO:1) is ligated into the p324 clone from which the Sal I to Xba I sequences described above
30 have been removed.

The resulting plasmid is designated BMP-9 fusion and comprises LINK-2, nucleotides #621-#1515 of murine BMP-9 (SEQ ID NO:1), nucleotides #35-#59 of LINK-1 (refer to the numbering of oligonucleotide #5), and the 374 bp Apa I/EcoR I fragment (human
35 BMP-9) derived from clone pBMP9mex-1 (described above) inserted between the Sal I and EcoR I sites of the mammalian cell expression vector pED6.

B. EXPRESSION

BMP-9 fusion is transfected into CHO cells using standard techniques known to those having ordinary skill in the art to create stable cell lines capable of expressing human BMP-9 protein. The cell lines are cultured under suitable culture conditions and the BMP-9 protein is isolated and purified from the culture medium.

In one embodiment, cells are grown in R1 medium based on a 50:50 mix of F12 and DME plus extra non-essential amino acids plus extra biotin and B12 and 10% fetal bovine serum (FBS) and 0.2 μ M methotrexate (MTX). Cells are grown up and expanded into roller bottles in this medium using confluent roller bottles. The serum containing growth medium is discarded, the rollers are rinsed with PBS-CMF, and a serum free production medium is added containing additional amino acids plus insulin (5 μ g/ml), putrescine (12.9 μ M), hydrocortisone (0.2 μ M), selenium (29 nM), and PVA (0.6 g/L). Dextran sulfate is used in this CM (at 100 μ g/ml). Conditioned medium (CM) is collected at 24 hours and the rollers are refed with fresh serum free medium. Four sequential 24 hour harvest can be collected. Conditioned medium is clarified (floating cells in the CM are removed) for purification by passing the CM through a 5 μ (pass Profile) pore size filter and a 0.22 μ (millipore Duropore) pore size filter.

EXAMPLE V**25 BIOLOGICAL ACTIVITY OF EXPRESSED BMP-9**

To measure the biological activity of the expressed BMP-9 proteins obtained in Example IV above, the proteins are recovered from the cell culture and purified by isolating the BMP-9 proteins from other proteinaceous materials with which they are co-produced as well as from other contaminants. The purified protein may be assayed in accordance with the rat bone formation assay described in Example III.

Purification is carried out using standard techniques known to those skilled in the art. It is contemplated, as with other BMP proteins, that purification may include the use of Heparin sepharose.

In one embodiment, 40 liters of the conditioned media from Example IV-B is titrated to pH 6.9 with concentrated sodium phosphate pH 6.0, and loaded onto Cellufine Sulfate, previously equilibrated with 50 mM sodium phosphate, pH 6.9. The resin is washed with 50 mM sodium phosphate, 0.5 M NaCl, followed by 50 mM sodium phosphate, 0.5 M NaCl, 0.5 M Arg, pH 6.9. BMP-9 is found in the wash as well as the elution, with a lesser amount of contaminants in the elution pool. Cellufine sulfate pools are concentrated and directly loaded onto RP-HPLC for final purification. Each concentrated pool is titrated to pH 3.8 with dilute TFA and loaded onto a 0.46 X 25 cm C₄ reverse phase column running a linear gradient from 30% A (0.1% TFA/H₂O) to 55% B (0.1% TFA/90% Acetonitrile) over 100 minutes. BMP-9 monomer is separated by baseline resolution from BMP-9 dimer. The identity of monomer and dimer pools are confirmed by N-terminal sequencing. Although heterogeneity in the N-terminus is expected sequencing reveals a predominant species Ser-Ala-Gly-Ala beginning with amino acid #1 of SEQ ID NO:9.

Protein analysis is conducted using standard techniques such as SDS-PAGE acrylamide [U.K. Laemmli, Nature 227:680 (1970)] stained with silver [R.R. Oakley et al., Anal. Biochem. 105:361 (1980)] and by immunoblot [H. Towbin et al., Proc. Natl. Acad. Sci. USA 76:4350 (1979)]. BMP-9 is efficiently expressed in CHO cells as a 14kDa nonglycosylated protein when analyzed under reducing conditions. BMP-9 is efficiently secreted within 4 hours of its synthesis.

EXAMPLE VI

A. W-20 BIOASSAY

Use of the W-20 bone marrow stromal cells as an indicator cell line is based upon the conversion of these cells to osteoblast-like cells after treatment with BMP-2 [R. S. Thies et al., "Bone Morphogenetic Protein alters W-20 stromal cell differentiation in vitro", Journal of Bone and Mineral Research 5(2):305 (1990); and R. S. Thies et al., "Recombinant Human Bone Morphogenetic Protein 2 Induces Osteoblastic Differentiation in W-20-17 Stromal Cells", Endocrinology, in press (1992)].

Specifically, W-20 cells are a clonal bone marrow stromal cell line derived from adult mice by researchers in the laboratory of Dr. D. Nathan, Children's Hospital, Boston, MA. BMP-2 treatment of W-20 cells results in (1) increased alkaline phosphatase production, (2) induction of PTH stimulated cAMP, and (3) induction of osteocalcin synthesis by the cells. While (1) and (2) represent characteristics associated with the osteoblast phenotype, the ability to synthesize osteocalcin is a phenotypic property only displayed by mature osteoblasts. Furthermore, to date we have observed conversion of W-20 stromal cells to osteoblast-like cells only upon treatment with BMPs. In this manner, the in vitro activities displayed by BMP treated W-20 cells correlate with the in vivo bone forming activity known for BMPs.

Below two in vitro assays useful in comparison of BMP activities of novel osteoinductive molecules are described.

B. W-20 ALKALINE PHOSPHATASE ASSAY PROTOCOL

W-20 cells are plated into 96 well tissue culture plates at a density of 10,000 cells per well in 200 μ l of media (DME with 10% heat inactivated fetal calf serum, 2 mM glutamine and 100 U/ml + 100 μ g/ml streptomycin. The cells are allowed to attach overnight in a 95% air, 5% CO₂ incubator at 37°C.

The 200 μ l of media is removed from each well with a multichannel pipettor and replaced with an equal volume of test sample delivered in DME with 10% heat inactivated fetal calf serum, 2 mM glutamine and 1% penicillin-streptomycin. Test substances are assayed in triplicate.

The test samples and standards are allowed a 24 hour incubation period with the W-20 indicator cells. After the 24 hours, plates are removed from the 37°C incubator and the test media are removed from the cells.

The W-20 cell layers are washed 3 times with 200 μ l per well of calcium/magnesium free phosphate buffered saline and these washes are discarded.

50 μ l of glass distilled water is added to each well and the assay plates are then placed on a dry ice/ethanol bath for quick

freezing. Once frozen, the assay plates are removed from the dry ice/ethanol bath and thawed at 37°C. This step is repeated 2 more times for a total of 3 freeze-thaw procedures. Once complete, the membrane bound alkaline phosphatase is available for measurement.

50 μ l of assay mix (50 mM glycine, 0.05% Triton X-100, 4 mM $MgCl_2$, 5 mM p-nitrophenol phosphate, pH = 10.3) is added to each assay well and the assay plates are then incubated for 30 minutes at 37°C in a shaking waterbath at 60 oscillations per minute.

At the end of the 30 minute incubation, the reaction is stopped by adding 100 μ l of 0.2 N NaOH to each well and placing the assay plates on ice.

The spectrophotometric absorbance for each well is read at a wavelength of 405 nanometers. These values are then compared to known standards to give an estimate of the alkaline phosphatase activity in each sample. For example, using known amounts of p-nitrophenol phosphate, absorbance values are generated. This is shown in Table I.

Table I
Absorbance Values for Known Standards
of P-Nitrophenol Phosphate

<u>P-nitrophenol phosphate umoles</u>	<u>Mean absorbance (405 nm)</u>
0.000	0
0.006	0.261 +/- .024
0.012	0.521 +/- .031
0.018	0.797 +/- .063
0.024	1.074 +/- .061
0.030	1.305 +/- .083

Absorbance values for known amounts of BMP-2 can be determined and converted to μ moles of p-nitrophenol phosphate cleaved per unit time as shown in Table II.

 Table II
 Alkaline Phosphatase Values for W-20 Cells
 Treating with BMP-2

	BMP-2 concentration ng/ml	Absorbance Reading 405 nmeters	umoles substrate per hour
5	0	0.645	0.024
	1.56	0.696	0.026
	3.12	0.765	0.029
10	6.25	0.923	0.036
	12.50	1.121	0.044
	25.0	1.457	0.058
	50.0	1.662	0.067
	100.0	1.977	0.080

15

 These values are then used to compare the activities of known amounts of BMP-9 to BMP-2.

C. OSTEOCALCIN RIA PROTOCOL

W-20 cells are plated at 10^6 cells per well in 24 well multiwell tissue culture dishes in 2 mls of DME containing 10% heat inactivated fetal calf serum, 2 mM glutamine. The cells are allowed to attach overnight in an atmosphere of 95% air 5% CO₂ at 37°C.

The next day the medium is changed to DME containing 10% fetal calf serum, 2 mM glutamine and the test substance in a total volume of 2 ml. Each test substance is administered to triplicate wells. The test substances are incubated with the W-20 cells for a total of 96 hours with replacement at 48 hours by the same test medias.

At the end of 96 hours, 50 μ l of the test media is removed from each well and assayed for osteocalcin production using a radioimmunoassay for mouse osteocalcin. The details of the assay are described in the kit manufactured by Biomedical Technologies Inc., 378 Page Street, Stoughton, MA 02072. Reagents for the assay are found as product numbers BT-431 (mouse osteocalcin standard), BT-432 (Goat anti-mouse Osteocalcin), BT-431R (iodinated mouse osteocalcin), BT-415 (normal goat serum) and BT-414 (donkey anti goat IgG). The RIA for osteocalcin synthesized by W-20 cells in response to BMP treatment is carried out as

described in the protocol provided by the manufacturer.

The values obtained for the test samples are compared to values for known standards of mouse osteocalcin and to the amount of osteocalcin produced by W-20 cells in response to challenge with known amounts of BMP-2.

Table III
Osteocalcin Synthesis by W-20 Cells

	<u>BMP-2 Concentration ng/ml</u>	<u>Osteocalcin Synthesis ng/well</u>
10	0	0.8
	2	0.9
	4	0.8
	8	2.2
	16	2.7
15	31	3.2
	62	5.1
	125	6.5
	250	8.2
	500	9.4
20	1000	10.0

EXAMPLE VII

ARTICULAR CARTILAGE ASSAY

The effect of BMP-9 on articular cartilage proteoglycan and DNA synthesis is assayed to determine if BMP-9 is involved in the regulation of metabolism of differentiated articular cartilage.

Articular cartilage explants from calf carpal joints are maintained in DMEM with 50 µg/ml ascorbate, 4 mM glutamine and antibiotics for 3 days. Cytokines (rhBMP-2, rhBMP-4, rhBMP-6 and rhBMP-9, IGF-1, bFGF (1-1000 ng/ml), and TGFβ (1-100 ng/ml)) are added to the medium and culture is continued for 3 more days. Medium is changed daily. Twenty-four hours prior to harvest, explants are pulsed with 50 µCi/ml ³⁵SO₄ or 25 µCi/ml ³H-thymidine. Explants are solubilized and separation of free isotope is performed by gel chromatography. Total DNA of each explant is measured by a spectrophotometric assay. BMP-9 stimulates proteoglycan synthesis above control levels at a dose of 10 ng/ml (p<0.05).

BMP-4, BMP-6, BMP-9 and TGFβ are significantly more active in stimulating proteoglycan synthesis at 100 ng/ml. At the

highest doses of cytokine tested (1 $\mu\text{g/ml}$), proteoglycan synthesis by explants exposed to all cytokines are significantly greater ($p < 0.05$) than that by control explants. Sulfate incorporation results are set forth in Figure 4.

5 Recombinant human BMP-9 stimulates alkaline phosphatase activity in the osteoprogenitor cell line, W-20-17, in a dose responsive manner with an ED_{50} of 4 ng/ml. In vivo, high doses are rhBMP-9 induce ectopic bone formation, with 25 $\mu\text{g/implant}$ of rhBMP-9 inducing cartilage and bone tissue after 10 days of
10 implantation.

EXAMPLE VIII

STIMULATION OF LIVER CELLS

It is contemplated that BMP-9 may be used in liver repair or regeneration. Through the use of whole embryo sections or
15 whole mount techniques, expression of mRNA in multiple tissue is screened simultaneously. In the 11.5 dpc mouse embryo, BMP-9 mRNA localizes exclusively to the developing liver. It is contemplated that BMP-9, like all other BMPs studied to date, acts as a local regulator of cell growth and differentiation,
20 therefore this very specific expression pattern suggests liver as a BMP-9 target tissue.

BMP-9 responsiveness in parenchymal liver cells is tested by screening four liver cell lines for their ability to bind iodinated, CHO-derived BMP-9. The four liver cell lines, HepG2
25 (ATCC HB8065), NMuLi (ATCC CRL1638), Chang and NCTC1469 (ATCC CCL9.1), all specifically bind ^{125}I -BMP-9 to some extent, with HepG2 and NCTC1469 cell lines exhibiting the highest degree of binding. Specific binding of BMP-9 to HepG2 cells is carried out by incubating HepG2 cells grown to confluence in Dulbecco's
30 Modified Eagle's Medium (DME) containing 10% heat-inactivated fetal calf serum (FCS) on gelatinized 6 well plates with 2 ng/ml ^{125}I -BMP-9 and increasing concentrations of unlabelled BMP-9 in binding buffer (136.9 mM NaCl, 5.37 mM KCl, 1.26 mM CaCl_2 , 0.64 mM MgSO_4 , 0.34 mM Na_2HPO_4 , 0.44 mM KH_2PO_4 , 0.49 mM MgCl_2 , 25 mM
35 HEPES and 0.5% BSA, pH 7.4) for 20 hours at 4°C to achieve binding equilibrium. This incubation follows a one hour

preincubation at 37°C in binding buffer alone. For crosslinking experiments, the cells were incubated with 500 μ M disuccinimidyl suberate for 20 minutes at 40°C following binding. Cell extracts were analyzed on SDS-PAGE. As shown in Figure 5, HepG2 cells expressed abundant high affinity receptors for BMP-9. Scatchard analysis of these binding data resulted in a curvilinear plot, with approximately 10,000 high affinity receptors per cell. These receptors exhibited a K_d of 0.3 nM. The curvilinear nature of the Scatchard plot indicates negative cooperativity among BMP-9 receptors or that HepG2 cells express at least two populations of BMP-9 receptors with different affinities. Crosslinking analysis on HepG2 cells with 125 I-BMP-9 yields two binding proteins of apparent molecular weights of 54 and 80 kD. Crosslinked ligand/receptor complexes were observed at 78 and 100 kD under nonreducing conditions, and 67 and 94 kD under reducing conditions. Subtracting the molecular weight of the BMP-9 dimer and monomer, respectively, it is estimated that these BMP-9 receptor proteins have molecular weights of approximately 54 and 80 kD. The K_d of the high affinity binding sites for BMP-9 is estimated to be approximately 270 pM for HepG2 cells. To test the binding specificity of the receptors for BMP-9, HepG2 cells were incubated with 125 I-BMP-9 and a 250-fold excess of different unlabeled ligands. The BMP-9 receptors expressed on HepG2 cells show only limited crossreactivity with BMPs 2 and 4, and no crossreactivity with BMPs 3, 5, 6, 7, 12 and 2/6, or with TGF- β 1 or TGF- β 2.

As a first indication of BMP-9 effects on confluent, serum starved HepG2 cells, cell proliferation is examined as determined by 3 H-thymidine incorporation and cell counting. HepG2 cells are plated at 10^6 cells/well in 96 well plates and cultured for 48 hours in DME/0.1% FCS to synchronize the cell cycle are treated for 24 hours with or without BMP-9 in the presence of 0.1% FCS. In 3 H-thymidine incorporation assays, 3 H-thymidine was included during the last 4 hours of treatment and cellular DNA was collected with a 96 well plate cell harvester. Proliferation was assayed by quantifying ethanol-precipitable 3 H-thymidine incorporation by liquid scintillation counting. For cell

counting assays, cells were trypsinized and counted with a hemacytometer. Primary rat hepatocytes isolated from male Fischer 344 rats (Charles River, Wilmington, MA) by collagenase digestion as perviously described [Michalopoulos et al., Cancer Res. 42:4673-4682 (1982)] are plated on collagen-coated plates at subconfluence (5,000-10,000 cells/cm²) in serum-free media as described in Michalopoulos et al., Cancer Res. 42:4673-4682 (1982) and treated with or without rhBMP-9 for 36 hours. ³H-thymidine was included throughout the treatment period and incorporated ³H-thymidine was quantified as described by Anscher et al., New England J. Med. 328:1592-1598 (1993). BMP-9 stimulates ³H-thymidine incorporation in HepG2 cells approximately five fold. This effect is confirmed by a stimulatory effect of BMP-9 in cell counting experiments. As shown in Figure 6, BMP-9 stimulated ³H-thymidine incorporation in HepG2 cells in a dose-responsive manner. The ED₅₀ for this effect was estimated at 10 ng/ml BMP-9. This ED₅₀ value is consistent with the estimated binding affinity (K_d = 0.3 nM = 8 ng/ml), suggesting that this biological effect is mediated by the described BMP-9 receptors.

To determine if this proliferative effect of BMP-9 was unique to the HepG2 liver tumor cell line, primary rat hepatocytes were tested for effects of BMP-9 on ³H-thymidine incorporated as shown in Figure 7. BMP-9 stimulated ³H-thymidine incorporation in primary hepatocytes, although not as markedly as EGF. This stimulatory effect is cell density-dependent in primary rat hepatocytes. While subconfluent cells exhibited a stimulation in response to BMP-9, confluent primary hepatocytes did not. As indicated in Figure 7, in contrast to rhBMP-9, TGF- β 1 was inhibitory, not stimulatory on primary rat hepatocytes.

The foregoing descriptions detail presently preferred embodiments of the present invention. Numerous modifications and variations in practice thereof are expected to occur to those skilled in the art upon consideration of these descriptions. Those modifications and variations are believed to be encompassed within the claims appended hereto.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Rosen, Vicki A.
Wozney, John M.
Celeste, Anthony J.
- (ii) TITLE OF INVENTION: BMP-9 COMPOSITIONS
- (iii) NUMBER OF SEQUENCES: 9
- 10 (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genetics Institute, Inc.
(B) STREET: Legal Affairs - 87 CambridgePark Drive
(C) CITY: Cambridge
(D) STATE: MA
15 (E) COUNTRY: US
(F) ZIP: 02140
- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
20 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:
- 25 (viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kapinos, Ellen J.
(B) REGISTRATION NUMBER: 32,245
(C) REFERENCE/DOCKET NUMBER: GI 5186C-PCT
- 30 (ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (617) 876-1210
(B) TELEFAX: (617) 876-5851

(2) INFORMATION FOR SEQ ID NO:1:

- 35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2447 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA to mRNA
- (iii) HYPOTHETICAL: NO
- 40 (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
(A) ORGANISM: Mus musculus
(B) STRAIN: C57B46xCBA
(F) TISSUE TYPE: liver
- 45 (vii) IMMEDIATE SOURCE:
(A) LIBRARY: Mouse liver cDNA
(B) CLONE: ML14A
- (viii) POSITION IN GENOME:
(C) UNITS: bp

(ix) FEATURE:
 (A) NAME/KEY: mat_peptide
 (B) LOCATION: 1564..1893

5 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 610..1896

(ix) FEATURE:
 (A) NAME/KEY: mRNA
 (B) LOCATION: 1..2447

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATGGCTATAC TTAGATTTAT GGATAGTTGG GTAGTAGGTG TAAATGTATG TGGTAAAAGG	180
CCTAGGAGAT TTGTTGATCC AATAAATATG ATTAGGGAAA CAATTATTAG GGTTCATGTT	240
15 CGTCCTTTTG GTGTGTGGAT TAGCATTATT TGTTTGATAA TAAGTTTAAC TAGTCAGTGT	300
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20 CCTGATGTTA GAAGGAGGGC TGAAAAGGCT CCTTCCCTCC CAGGACAAAA CCGGAGCAGG	600
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25 TTC CTG CTG GTC TGT GTC ACA CAG CAG AAG CCG CTG CAG AAC TGG GAA	696
Phe Leu Leu Val Cys Val Thr Gln Gln Lys Pro Leu Gln Asn Trp Glu	
-305 -300 -295 -290	
CAA GCA TCC CCT GGG GAA AAT GCC CAC AGC TCC CTG GGA TTG TCT GGA	744
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35 AAG GTG GAT TTC CTA CGC AGC CTT AAC CTC AGC GGC ATT CCC TCC CAG	840
Lys Val Asp Phe Leu Arg Ser Leu Asn Leu Ser Gly Ile Pro Ser Gln	
-255 -250 -245	
GAC AAA ACC AGA GCG GAG CCA CCC CAG TAC ATG ATC GAC TTG TAC AAC	888
Asp Lys Thr Arg Ala Glu Pro Pro Gln Tyr Met Ile Asp Leu Tyr Asn	
-240 -235 -230	
40 AGA TAC ACA ACG GAC AAA TCG TCT ACG CCT GCC TCC AAC ATC GTG CGG	936
Arg Tyr Thr Thr Asp Lys Ser Ser Thr Pro Ala Ser Asn Ile Val Arg	
-225 -220 -215 -210	
AGC TTC AGC GTG GAA GAT GCT ATA TCG ACA GCT GCC ACG GAG GAC TTC	984
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-205 -200 -195	

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5	GAG CAG ATC ACC AGG GCT GAG CTC CGA CTC TAT GTC TCC TGC CAA AAT Glu Gln Ile Thr Arg Ala Glu Leu Arg Leu Tyr Val Ser Cys Gln Asn -175 -170 -165	1080
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10	GTT CTG GAG GAC AGT GAG ACT TGG GAC CAG GCC ACG GGG ACC AAG ACC Val Leu Glu Asp Ser Glu Thr Trp Asp Gln Ala Thr Gly Thr Lys Thr -145 -140 -135	1176
15	TTC TTG GTA TCC CAG GAC ATT CGG GAC GAA GGA TGG GAG ACT TTA GAA Phe Leu Val Ser Gln Asp Ile Arg Asp Glu Gly Trp Glu Thr Leu Glu -125 -120 -115	1224
	GTA TCG AGT GCC GTG AAG CGG TGG GTC AGG GCA GAC TCC ACA ACA AAC Val Ser Ser Ala Val Lys Arg Trp Val Arg Ala Asp Ser Thr Thr Asn -110 -105 -100	1272
20	AAA AAT AAG CTC GAG GTG ACA GTG CAG AGC CAC AGG GAG AGC TGT GAC Lys Asn Lys Leu Glu Val Thr Val Gln Ser His Arg Glu Ser Cys Asp -95 -90 -85	1320
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30	GAG CTG AAG GAG ATG ATC GGC CAT GAG CAG GAG ACC ATG CTT GTG AAG Glu Leu Lys Glu Met Ile Gly His Glu Gln Glu Thr Met Leu Val Lys -45 -40 -35	1464
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35	GGT CTA GAT GGA TAC ACA GCT GTG GGA CCA CTT TTA GCT AGA AGG AAG Gly Leu Asp Gly Tyr Thr Ala Val Gly Pro Leu Leu Ala Arg Arg Lys -15 -10 -5	1560
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	GAC GTG ACA CCC ACC AAA CAT GCC ATC GTG CAG ACC CTG GTG CAT CTC Asp Val Thr Pro Thr Lys His Ala Ile Val Gln Thr Leu Val His Leu 50 55 60	1752
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5 AAG TAC CAC TAT GAG GGG ATG AGT GTG GCT GAG TGT GGG TGT AGG TAGTCCCTGC 1903
 Lys Tyr His Tyr Glu Gly Met Ser Val Ala Glu Cys Gly Cys Arg
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 10 CCTTCTTGTG TCTGGTGGGT CCCTCTGCTG AAGTGACAAT GACTGGGGTA TGCGGGCCTG 2083
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 TGTCTCAGG GAGAACAGCA TTGCTGTTCC TGTGCCTCAA GCTCCAGCT GACTCTCCTG 2323
 15 TGGCTCATAG GACTGAATGG GGTGAGGAAG AGCCTGATGC CCTCTGGCAA TCAGAGCCCCG 2383
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
 20 (A) LENGTH: 428 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

25 Met Ser Pro Gly Ala Phe Arg Val Ala Leu Leu Pro Leu Phe Leu Leu
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30 Pro Gly Glu Asn Ala His Ser Ser Leu Gly Leu Ser Gly Ala Gly Glu
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 Glu Gly Val Phe Asp Leu Gln Met Phe Leu Glu Asn Met Lys Val Asp
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 -250 -245 -240

35 Arg Ala Glu Pro Pro Gln Tyr Met Ile Asp Leu Tyr Asn Arg Tyr Thr
 -235 -230 -225
 Thr Asp Lys Ser Ser Thr Pro Ala Ser Asn Ile Val Arg Ser Phe Ser
 -220 -215 -210

40 Val Glu Asp Ala Ile Ser Thr Ala Ala Thr Glu Asp Phe Pro Phe Gln
 -205 -200 -195
 Lys His Ile Leu Ile Phe Asn Ile Ser Ile Pro Arg His Glu Gln Ile
 -190 -185 -180 -175

Thr Arg Ala Glu Leu Arg Leu Tyr Val Ser Cys Gln Asn Asp Val Asp
 -170 -165 -160
 Ser Thr His Gly Leu Glu Gly Ser Met Val Val Tyr Asp Val Leu Glu
 -155 -150 -145
 5 Asp Ser Glu Thr Trp Asp Gln Ala Thr Gly Thr Lys Thr Phe Leu Val
 -140 -135 -130
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 -125 -120 -115
 10 Ala Val Lys Arg Trp Val Arg Ala Asp Ser Thr Thr Asn Lys Asn Lys
 -110 -105 -100 -95
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 -90 -85 -80
 Ile Ser Val Pro Pro Gly Ser Lys Asn Leu Pro Phe Phe Val Val Phe
 -75 -70 -65
 15 Ser Asn Asp Arg Ser Asn Gly Thr Lys Glu Thr Arg Leu Glu Leu Lys
 -60 -55 -50
 Glu Met Ile Gly His Glu Gln Glu Thr Met Leu Val Lys Thr Ala Lys
 -45 -40 -35
 20 Asn Ala Tyr Gln Val Ala Gly Glu Ser Gln Glu Glu Gly Leu Asp
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 5 10 15
 25 Asp Ile Gly Trp Asp Ser Trp Ile Ile Ala Pro Lys Glu Tyr Asp Ala
 20 25 30
 Tyr Glu Cys Lys Gly Gly Cys Phe Phe Pro Leu Ala Asp Asp Val Thr
 35 40 45 50
 30 Pro Thr Lys His Ala Ile Val Gln Thr Leu Val His Leu Glu Phe Pro
 55 60 65
 Thr Lys Val Gly Lys Ala Cys Cys Val Pro Thr Lys Leu Ser Pro Ile
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 Ser Ile Leu Tyr Lys Asp Asp Met Gly Val Pro Thr Leu Lys Tyr His
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 35 Tyr Glu Gly Met Ser Val Ala Glu Cys Gly Cys Arg
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(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1954 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: cDNA to mRNA
 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: Homo sapiens
 (G) CELL TYPE: Osteosarcoma Cell Line
 (H) CELL LINE: U-2OS

(vii) IMMEDIATE SOURCE:
 (A) LIBRARY: U2OS cDNA in Lambda gt10
 (B) CLONE: Lambda U2OS-3

(viii) POSITION IN GENOME:
 (C) UNITS: bp

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 403..1629

(ix) FEATURE:
 (A) NAME/KEY: mat peptide
 (B) LOCATION: 1275..1626

(ix) FEATURE:
 (A) NAME/KEY: mRNA
 (B) LOCATION: 9..1934

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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CTGGCGAGCC	CGCTACTGCA	GGGACCTATG	GAGCCATTCC	GTAGTGCCAT	CCCGAGCAAC	300
GCACTGCTGC	AGCTTCCCTG	AGCCTTTCCA	GCAAGTTTGT	TCAAGATTGG	CTGTCAAGAA	360
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Asn Arg Met	Leu Met Val	Val Leu Leu	Cys Gln Val	Leu Leu Gly	Gly	
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GAG ATT CAG	GGC CAC GCG	GGA GGA CGC	CGC TCA GGG	CAG AGC CAT	GAG	558
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	-255	-250		-245		
CTC CTG CGG	GAC TTC GAG	GCG ACA CTT	CTG CAG ATG	TTT GGG CTG	CGC	606
Leu Leu Arg	Asp Phe Glu	Ala Thr Leu	Leu Gln Met	Phe Gly Leu	Arg	
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CGC CGC CCG	CAG CCT AGC	AAG AGT GCC	GTC ATT CCG	GAC TAC ATG	CGG	654
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10	AGT GAA AAC TCT GCT TTT CGT TTC CTC TTT AAC CTC AGC AGC ATC CCT	846
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	GAG AAC GAG GTG ATC TCC TCT GCA GAG CTT CGG CTC TTC CGG GAG CAG	894
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	Val Asp Gln Gly Pro Asp Trp Glu Arg Gly Phe His Arg Ile Asn Ile	
	-125 -120 -115	
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	ACA CGA CTA CTG GAC ACG AGA CTG GTC CAC CAC AAT GTG ACA CGG TGG	1038
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45	Arg Arg His Ser Leu Tyr Val Asp Phe Ser Asp Val Gly Trp Asn Asp	
	20 25 30	
	TGG ATT GTG GCC CCA CCA GGC TAC CAG GCC TTC TAC TGC CAT GGC GAC	1422
	Trp Ile Val Ala Pro Pro Gly Tyr Gln Ala Phe Tyr Cys His Gly Asp	
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50	TGC CCC TTT CCA CTG GCT GAC CAC CTC AAC TCA ACC AAC CAT GCC ATT	1470
	Cys Pro Phe Pro Leu Ala Asp His Leu Asn Ser Thr Asn His Ala Ile	
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GTG CAG ACC CTG GTC AAT TCT GTC AAT TCC AGT ATC CCC AAA GCC TGT 1518
 Val Gln Thr Leu Val Asn Ser Val Asn Ser Ser Ile Pro Lys Ala Cys
 65 70 75 80
 5 TGT GTG CCC ACT GAA CTG AGT GCC ATC TCC ATG CTG TAC CTG GAT GAG 1566
 Cys Val Pro Thr Glu Leu Ser Ala Ile Ser Met Leu Tyr Leu Asp Glu
 85 90 95
 TAT GAT AAG GTG GTA CTG AAA AAT TAT CAG GAG ATG GTA GTA GAG GGA 1614
 Tyr Asp Lys Val Leu Lys Asn Tyr Gln Glu Met Val Val Glu Gly
 100 105 110
 10 TGT GGG TGC CGC TGAGATCAGG CAGTCCTTGA GGATAGACAG ATATACACAC 1666
 Cys Gly Cys Arg
 115
 CACACACACA CACCACATAC ACCACACACA CACGTTCCCA TCCACTCACC CACACACTAC 1726
 ACAGACTGCT TCCTTATAGC TGGACTTTTA TTTAAAAAAA AAAAAAAAAA AATGGAAAAA 1786
 15 ATCCCTAAAC ATTCACCTTG ACCTTATTTA TGACTTTACG TGCAAATGTT TTGACCATAT 1846
 TGATCATATA TTTTGACAAA ATATATTTAT AACTACGTAT TAAAAGAAAA AAATAAAATG 1906
 AGTCATTATT TTAATAAAAA AAAAAAACT CTAGAGTCGA CGGAATTC 1954

(2) INFORMATION FOR SEQ ID NO:4:

20 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 408 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

25 Met Ile Pro Gly Asn Arg Met Leu Met Val Val Leu Leu Cys Gln Val
 -292 -290 -285 -280
 Leu Leu Gly Gly Ala Ser His Ala Ser Leu Ile Pro Glu Thr Gly Lys
 -275 -270 -265
 30 Lys Lys Val Ala Glu Ile Gln Gly His Ala Gly Gly Arg Arg Ser Gly -245
 -260 -255 -250
 Gln Ser His Glu Leu Leu Arg Asp Phe Glu Ala Thr Leu Leu Gln Met
 -240 -235 -230
 Phe Gly Leu Arg Arg Arg Pro Gln Pro Ser Lys Ser Ala Val Ile Pro
 -225 -220 -215
 35 Asp Tyr Met Arg Asp Leu Tyr Arg Leu Gln Ser Gly Glu Glu Glu
 -210 -205 -200
 Glu Gln Ile His Ser Thr Gly Leu Glu Tyr Pro Glu Arg Pro Ala Ser
 -195 -190 -185
 40 Arg Ala Asn Thr Val Arg Ser Phe His His Glu Glu His Leu Glu Asn -165
 -180 -175 -170
 Ile Pro Gly Thr Ser Glu Asn Ser Ala Phe Arg Phe Leu Phe Asn Leu
 -160 -155 -150
 Ser Ser Ile Pro Glu Asn Glu Val Ile Ser Ser Ala Glu Leu Arg Leu
 -145 -140 -135

Phe Arg Glu Gln Val Asp Gln Gly Pro Asp Trp Glu Arg Gly Phe His
 -130 -125 -120
 Arg Ile Asn Ile Tyr Glu Val Met Lys Pro Pro Ala Glu Val Val Pro
 -115 -110 -105
 5 Gly His Leu Ile Thr Arg Leu Leu Asp Thr Arg Leu Val His His Asn
 -100 -95 -90 -85
 Val Thr Arg Trp Glu Thr Phe Asp Val Ser Pro Ala Val Leu Arg Trp
 -80 -75 -70
 10 Thr Arg Glu Lys Gln Pro Asn Tyr Gly Leu Ala Ile Glu Val Thr His
 -65 -60 -55
 Leu His Gln Thr Arg Thr His Gln Gly Gln His Val Arg Ile Ser Arg
 -50 -45 -40
 Ser Leu Pro Gln Gly Ser Gly Asn Trp Ala Gln Leu Arg Pro Leu Leu
 -35 -30 -25
 15 Val Thr Phe Gly His Asp Gly Arg Gly His Ala Leu Thr Arg Arg Arg
 -20 -15 -10 -5
 Arg Ala Lys Arg Ser Pro Lys His His Ser Gln Arg Ala Arg Lys Lys
 1 5 10
 20 Asn Lys Asn Cys Arg Arg His Ser Leu Tyr Val Asp Phe Ser Asp Val
 15 20 25
 Gly Trp Asn Asp Trp Ile Val Ala Pro Pro Gly Tyr Gln Ala Phe Tyr
 30 35 40
 Cys His Gly Asp Cys Pro Phe Pro Leu Ala Asp His Leu Asn Ser Thr
 45 50 55 60
 25 Asn His Ala Ile Val Gln Thr Leu Val Asn Ser Val Asn Ser Ser Ile
 65 70 75
 Pro Lys Ala Cys Cys Val Pro Thr Glu Leu Ser Ala Ile Ser Met Leu
 80 85 90
 30 Tyr Leu Asp Glu Tyr Asp Lys Val Val Leu Lys Asn Tyr Gln Glu Met
 95 100 105
 Val Val Glu Gly Cys Gly Cys Arg
 110 115

(2) INFORMATION FOR SEQ ID NO:5:

- 35 (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 15 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: cDNA to mRNA
 40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CATGGGCAGC TCGAG

15

(2) INFORMATION FOR SEQ ID NO:6:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 34 base pairs

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTGCAGGCCGA GCCTGAATTC CTCGAGCCAT CATG

34

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 68 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: cDNA to mRNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

15 CGAGGTAA A AACGTCTAG GCCCCCCGAA CCACGGGGAC GTGGTTTCC TTTGAAAAAC
ACGATTGC

60

68

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 470 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

25

(v) FRAGMENT TYPE: C-terminal

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens
- (H) CELL LINE: W138 (genomic DNA)

(vii) IMMEDIATE SOURCE:

30

- (A) LIBRARY: human genomic library
- (B) CLONE: lambda 111-1

(viii) POSITION IN GENOME:

(C) UNITS: bp

(ix) FEATURE:

35

- (A) NAME/KEY: exon
- (B) LOCATION: 1..470

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..456

40

(ix) FEATURE:

- (A) NAME/KEY: mat_peptide
- (B) LOCATION: 124..453

(ix) FEATURE:

45

- (A) NAME/KEY: mRNA
- (B) LOCATION: 1..470

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

	TGA	ACA	AGA	GAG	TGC	TCA	AGA	AGC	TGT	CCA	AGG	ACG	GCT	CCA	CAG	AGG	48
	Thr	Arg	Glu	Cys	Ser	Arg	Ser	Cys	Pro	Arg	Thr	Ala	Pro	Gln	Arg		
	-41	-40				-35					-30						
5	CAG	GTG	AGA	GCA	GTC	ACG	AGG	AGG	ACA	CGG	ATG	GCG	CAC	GTG	GCT	GCG	96
	Gln	Val	Arg	Ala	Val	Thr	Arg	Arg	Thr	Arg	Met	Ala	His	Val	Ala	Ala	
	-25				-20					-15					-10		
	GGG	TCG	ACT	TTA	GCC	AGG	CGG	AAA	AGG	AGC	GCC	GGG	GCT	GGC	AGC	CAC	144
10	Gly	Ser	Thr	Leu	Ala	Arg	Arg	Lys	Arg	Ser	Ala	Gly	Ala	Gly	Ser	His	
				-5						1				5			
	TGT	CAA	AAG	ACC	TCC	CTG	CGG	GTA	AAC	TTC	GAG	GAC	ATC	GGC	TGG	GAC	192
	Cys	Gln	Lys	Thr	Ser	Leu	Arg	Val	Asn	Phe	Glu	Asp	Ile	Gly	Trp	Asp	
			10					15					20				
	AGC	TGG	ATC	ATT	GCA	CCC	AAG	GAG	TAT	GAA	GCC	TAC	GAG	TGT	AAG	GGC	240
15	Ser	Trp	Ile	Ile	Ala	Pro	Lys	Glu	Tyr	Glu	Ala	Tyr	Glu	Cys	Lys	Gly	
	25					30					35						
	GGC	TGC	TTC	TTC	CCC	TTG	GCT	GAC	GAT	GTG	ACG	CCG	ACG	AAA	CAC	GCT	288
	Gly	Cys	Phe	Phe	Pro	Leu	Ala	Asp	Asp	Val	Thr	Pro	Thr	Lys	His	Ala	
	40					45				50					55		
20	ATC	GTG	CAG	ACC	CTG	GTG	CAT	CTC	AAG	TTC	CCC	ACA	AAG	GTG	GGC	AAG	336
	Ile	Val	Gln	Thr	Leu	Val	His	Leu	Lys	Phe	Pro	Thr	Lys	Val	Gly	Lys	
					60					65					70		
	GCC	TGC	TGT	GTG	CCC	ACC	AAA	CTG	AGC	CCC	ATC	TCC	GTC	CTC	TAC	AAG	384
25	Ala	Cys	Cys	Val	Pro	Thr	Lys	Leu	Ser	Pro	Ile	Ser	Val	Leu	Tyr	Lys	
				75					80					85			
	GAT	GAC	ATG	GGG	GTG	CCC	ACC	CTC	AAG	TAC	CAT	TAC	GAG	GGC	ATG	AGC	432
	Asp	Asp	Met	Gly	Val	Pro	Thr	Leu	Lys	Tyr	His	Tyr	Glu	Gly	Met	Ser	
		90						95					100				
30	GTG	GCA	GAG	TGT	GGG	TGC	AGG	TAGTATCTGC	CTGCGGG								470
	Val	Ala	Glu	Cys	Gly	Cys	Arg										
	105					110											

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 150 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

40	Thr	Arg	Glu	Cys	Ser	Arg	Ser	Cys	Pro	Arg	Thr	Ala	Pro	Gln	Arg	
	-41	-40				-35					-30					
	Gln	Val	Arg	Ala	Val	Thr	Arg	Arg	Thr	Arg	Met	Ala	His	Val	Ala	Ala
	-25				-20					-15					-10	
	Gly	Ser	Thr	Leu	Ala	Arg	Arg	Lys	Arg	Ser	Ala	Gly	Ala	Gly	Ser	His
				-5					1					5		
45	Cys	Gln	Lys	Thr	Ser	Leu	Arg	Val	Asn	Phe	Glu	Asp	Ile	Gly	Trp	Asp
		10						15					20			

Ser Trp Ile Ile Ala Pro Lys Glu Tyr Glu Ala Tyr Glu Cys Lys Gly
25 30 35
Gly Cys Phe Phe Pro Leu Ala Asp Asp Val Thr Pro Thr Lys His Ala
40 45 50 55
5 Ile Val Gln Thr Leu Val His Leu Lys Phe Pro Thr Lys Val Gly Lys
60 65 70
Ala Cys Cys Val Pro Thr Lys Leu Ser Pro Ile Ser Val Leu Tyr Lys
75 80 85
10 Asp Asp Met Gly Val Pro Thr Leu Lys Tyr His Tyr Glu Gly Met Ser
90 95 100
Val Ala Glu Cys Gly Cys Arg
105 110

What is claimed is:

1. A BMP-9 polypeptide comprising the amino acid sequence from amino acid #8 - 110 as set forth in FIG. 3 (SEQ ID NO: 9).
2. A BMP-9 polypeptide comprising the amino acid sequence from amino acid #1 - 110 as set forth in FIG. 3 (SEQ ID NO: 9).
3. A BMP-9 polypeptide of claim 1 wherein said polypeptide is a dimer wherein each subunit comprises at least the amino acid sequence from amino acid #8 - 110 of FIG. 3 (SEQ ID NO: 9).
4. A BMP-9 polypeptide of claim 2 wherein said polypeptide is a dimer wherein each subunit comprises at least the amino acid sequence from amino acid #1-110 of FIG. 3. (SEQ ID NO: 9).
5. A purified BMP-9 protein produced by the steps of
 - (a) culturing a cell transformed with a cDNA comprising the nucleotide sequence from nucleotide #124 to #453 as shown in FIG. 3 (SEQ ID NO: 8); and
 - (b) recovering and purifying from said culture medium a protein comprising the amino acid sequence from amino acid #1 to amino acid #110 as shown in FIG. 3 (SEQ ID NO: 9).
6. A purified BMP-9 protein produced by the steps of
 - (a) culturing a cell transformed with a cDNA comprising the nucleotide sequence from nucleotide #124 to #453 as shown in FIG. 3 (SEQ ID NO: 8); and
 - (b) recovering from said culture medium a protein comprising an amino acid sequence from amino acid #8 to amino acid #110 as shown in Figure 3 (SEQ ID NO: 9).
7. A BMP-9 protein characterized by the ability to induce the formation of cartilage and/or bone.
8. A DNA sequence encoding a BMP-9 protein.

9. The DNA sequence of claim 8 wherein said DNA comprises
(a) nucleotide 124 to 453 (SEQ ID NO: 8); and
(b) sequences which hybridize thereto under stringent
hybridization conditions and exhibit the ability to form
cartilage and/or bone.
10. The DNA sequence of claim 8 wherein said DNA comprises
(a) nucleotide 145 to 453 (SEQ ID NO: 8); and
(b) sequences which hybridize thereto under stringent
hybridization conditions and exhibit the ability to form
cartilage and/or bone.
11. A host cell transformed with a DNA sequence encoding BMP-8.
12. A method for producing a purified BMP-9 protein said method
comprising the steps of
(a) culturing a cell transformed with a cDNA comprising the
nucleotide sequence encoding a BMP-9 protein; and
(b) recovering and purifying said BMP-9 protein from the
culture medium.
13. A pharmaceutical composition comprising an effective amount
of a BMP-9 protein in admixture with a pharmaceutically
acceptable vehicle.
14. A composition of claim 13 further comprising a matrix for
supporting said composition and providing a surface for bone
and/or cartilage growth.
15. The composition of claim 14 wherein said matrix comprises
a material selected from the group consisting of hydroxyapatite,
collagen, polylactic acid and tricalcium phosphate.
16. A method for inducing bone and/or cartilage formation in a
patient in need of same comprising administering to said patient
an effective amount of the composition of claim 13.

17. A pharmaceutical composition for wound healing and tissue repair said composition comprising an effective amount of a BMP-9 protein in a pharmaceutically acceptable vehicle.

5 18. A method for treating wounds and/or tissue repair in a patient in need of same comprising administering to said patient an effective amount of the composition of claim 17.

19. A purified mammalian BMP-9 protein produced by the steps of
10 (a) culturing a cell transformed with (i) a DNA comprising the nucleotide sequence from nucleotide #610 to #1893 of SEQ ID NO:1 and (ii) sequences which hybridize thereto under stringent hybridization conditions and induces the formation of cartilage or bone; and

(b) recovering and purifying from said culture medium a protein comprising amino acid #1 to #110 of SEQ ID NO:9.

15 20. A pharmaceutical composition for hepatocyte growth said composition comprising an effective amount of a BMP-9 protein in a pharmaceutically acceptable vehicle.

21. A method for inducing hepatocyte growth in a patient in need of same comprising administering to said patient an effective
20 amount of the composition of claim 20.

22. A pharmaceutical composition for cartilage repair said composition comprising an effective amount of a BMP-9 protein in a pharmaceutically acceptable vehicle.

Figure 1/1

10	20	30	40	50	60	70
CATTAATAAA	TATTAAGTAT	TGGAATTAGT	GAAATTGGAG	TTCCCTTGTGG	AAGGAAGTGG	GCAAGTGAGC
80	90	100	110	120	130	140
TTTTTAGTTT	GTGTCGGAAG	CCTGTAAATTA	CGGCTCCAGC	TCATAGTGGA	ATGGCTATAC	TTAGATTATAT
150	160	170	180	190	200	210
GGATAGTTGG	GTAGTAGGTG	TAAATGTATG	TGGTAAAAGG	CCTAGGAGAT	TTGTTGATCC	AATAAATATG
220	230	240	250	260	270	280
ATTAGGAAA	CAATTATTAG	GGTTCATGTT	CGTCCCTTTTG	GTGTGTGGAT	TAGCATTATT	TGTTTGATAA
290	300	310	320	330	340	350
TAAAGTTAAC	TAGTCAGTGT	TGGAAGAAT	GGAGACGGTT	GTTGATTAGG	CGTTTTGAGG	ATGGAATAG
360	370	380	390	400	410	420
GATTGAAGGA	AATATAATGA	TGGCTACAC	GATTGGGAAT	CCTATTATTG	TTGGGGTAAT	GAATGAGGCA
430	440	450	460	470	480	490
AATAGATTTT	CGTTTCATTTT	AATTCTCAAG	GGGTTTTTTAC	TTTTTATGTTT	GTTAGTGATA	TTGGTGAGTA
500	510	520	530	540	550	560
GGCCAAGGGT	TAATAGTGTA	ATTGAATTAT	AGTGAATCA	TATTACTAGA	CCTGATGTTA	GAAGGAGGGC
570	580	590	600	609	618	

> _____
M S P G

TGAAAAGGCT CCTTCCCTCC CAGGACAAA CCGGAGCAGG GCCACCGG ATG TCC CCT GGG

 M S P G

Figure 1/2

627	636	645	654	663	672
GCC TTC CGG GTG GCC CTG CTC CCG CTG TTC CTG GTC TGT GTC ACA CAG CAG					
A F R V A L L P L F L L V C V T Q Q					
681	690	699	708	717	726
AAG CCG CTG CAG AAC TGG GAA CAA GCA TCC CCT GGG GAA AAT GCC CAC AGC TCC					
K P L Q N W E Q A S P G E N A H S S					
735	744	753	762	771	780
CTG GGA TTG TCT GGA GCT GGA GAG GAG GGT GTC TTT GAC CTG CAG ATG TTC CTG					
L G L S G A G E E G V F D L Q M F L					
789	798	807	816	825	834
GAG AAC ATG AAG GTG GAT TTC CTA CGC AGC CTT AAC CTC AGC GGC ATT CCC TCC					
E N M K V D F L R S L N L S G I P S					

Figure 1/3

843	852	861	870	879	888
CAG GAC AAA ACC AGA GCG GAG CCA CCC CAG TAC ATG ATC GAC TTG TAC AAC AGA					
Q D K T R A E P P Q Y M I D L Y N R					
897	906	915	924	933	942
TAC ACA ACG GAC AAA TCG TCT ACG CCT GCC TCC AAC ATC GTG CGG AGC TTC AGC					
Y T T D K S S T P A S N I V R S F S					
951	960	969	978	987	996
GTG GAA GAT GCT ATA TCG ACA GCT GCC ACG GAG GAC TTC CCC TTT CAG AAG CAC					
V E D A I S T A A T E D F P F Q K H					
1005	1014	1023	1032	1041	1050
ATC CTG ATC TTC AAC ATC TCC ATC CCG AGG CAC GAG CAG ATC ACC AGG GCT GAG					
I L I F N I S I P R H E Q I T R A E					

Figure 1/4

1059	1068	1077	1086	1095	1104
CTC CGA CTC TAT GTC TCC TGC CAA AAT GAT GTG GAC TCC ACT CAT GGG CTG GAA					
L R L Y V S C Q N D V D S T H G L E					
1113	1122	1131	1140	1149	1158
CGA AGC ATG GTC GTT TAT GAT GTT CTG GAG GAC AGT GAG ACT TGG GAC CAG GCC					
G S M V V Y D V L E D S E T W D Q A					
1167	1176	1185	1194	1203	1212
ACG GGG ACC AAG ACC TTC TTG GTA TCC CAG GAC ATT CGG GAC GAA GGA TGG GAG					
T G T K T P L V S Q D I R D E G W E					
1221	1230	1239	1248	1257	1266
ACT TTA GAA GTA TCG AGT GCC GTG AAG CGG TGG GTC AGG GCA GAC TCC ACA ACA					
T L E V S S A V K R W V R A D S T T					

Figure 1/5

1275	1284	1293	1302	1311	1320
AAC AAA AAT AAG CTC GAG GTG ACA GTG CAG AGC CAC AGG GAG AGC TGT GAC ACA					
N K N K L E V T V Q S H R E S C D T					
1329	1338	1347	1356	1365	1374
CTG GAC ATC AGT GTC CCT CCA GGT TCC AAA AAC CTG CCC TTC TTT GTT GTC TTC					
L D I S V P P G S K N L P F F V V F					
1383	1392	1401	1410	1419	1428
TCC AAT GAC CGC AGC AAT GGG ACC AAG GAG ACC AGA CTG GAG CTG AAG GAG ATG					
S N D R S N G T K E T R L E L K E M					
1437	1446	1455	1464	1473	1482
ATC GGC CAT GAG CAG GAG ACC ATG CTT GTG AAG ACA GCC AAA AAT GCT TAC CAG					
I G H E Q E T M L V K T A K N A Y Q					

Figure 1/6

1491	1500	1509	1518	1527	1536
GTG GCA GGT GAG AGC CAA GAG GAG GAG GGT CTA GAT GGA TAC ACA GCT GTG GGA					
V A G E S Q E E E G L D G Y T A V G					
1545	1554	1563	1572	1581	1590
CCA CTT TTA GCT AGA AGG AAG AGG AGC ACC GGA GCC AGC AGC CAC TGC CAG AAG					
P L L A R R K R S T G A S S H C Q K					
		(319)			(326)
1599	1608	1617	1626	1635	1644
ACT TCT CTC AGG GTG AAC TTT GAG GAC ATC GGC TGG GAC AGC TGG ATC ATT GCA					
T S L R V N F E D I G W D S W I I A					
1653	1662	1671	1680	1689	1698
CCC AAG GAA TAT GAC GCC TAT GAG TGT AAA GGG GGT TGC TTC TTC CCA TTG GCT					
P K E Y D A Y E C K G G G C F F P L A					

Figure 1/7

1707	1716	1725	1734	1743	1752
GAT GAC GTG ACA CCC ACC AAA CAT GCC ATC GTG CAG ACC CTG GTG CAT CTC GAG					
D D V T P T K H A I V Q T L V H L E					
1761	1770	1779	1788	1797	1806
TTC CCC ACA AAG GTG GGC AAA GCC TGC TGC GTT CCC ACC AAA CTG AGT CCC ATC					
F P T K V G K A C C V P T K L S P I					
1815	1824	1833	1842	1851	1860
TCC ATC CTC TAC AAG GAT GAC ATG GGG GTG CCA ACC CTC AAG TAC CAC TAT GAG					
S I L Y K D D M G V P T L K Y H Y E					
1869	1878	1887	1903	1913	1923
GGG ATG AGT GTG GCT GAG TGT GGG TGT AGG TAGTCCCTGC AGCCACCAG GGTGGGGATA					
G M S V A E C G C R					

(428)

Figure 1/8

1933	1943	1953	1963	1973	1983	1993
CAGGACATGG	AAGAGGTTCT	GGTACGGTCC	TGCATCCTCC	TGCGCATGGT	ATGCCCTAAGT	TGATCAGAAA
2003	2013	2023	2033	2043	2053	2063
CCATCCTTGA	GAAAGAAAAGG	AGTTAGTTGC	CCTTCTTGTG	TCTGGTGGGT	CCCTCTGCTG	AAGTGACAAT
2073	2083	2093	2103	2113	2123	2133
GACTGGGGTA	TGCGGGCCTG	TGGGCAGAGC	AGGAGACCCT	GGAAGGGTTA	GTGGGTAGAA	AGATGTCAAA
2143	2153	2163	2173	2183	2193	2203
AAGGAAGCTG	TGGGTAGATG	ACCTGCACTC	CAGTGATTAG	AAGTCCAGCC	TTACCTGTGA	GAGAGCTCCT
2213	2223	2233	2243	2253	2263	2273
GGCATCTAAG	AGAACTCTGC	TTCCCTCATCA	TCCCCACCGA	CTTGTTCTTC	CTTGGGAGTG	TGTCCTCAGG
2283	2293	2303	2313	2323	2333	2343
GAGAACAGCA	TTGCTGTTCC	TGTGCCCTCAA	GCTCCCAGCT	GACTCTCCTG	TGGCTCATAG	GACTGAATGG
2353	2363	2373	2383	2393	2403	2413
GGTGAGGAAG	AGCCTGATGC	CCTCTGGCAA	TCAGAGCCCG	AAGGACTTCA	AAACATCTGG	ACAACTCTCA
2423	2433	2443				
TTGACTGATG	CTCCAACATA	ATTTTAAAA	AGAG			

Figure 2/1

10	20	30	40	50	60	70
CTCTAGAGG	CAGAGGAGGA	GGGAGGAGG	GAAGGAGCG	GGAGCCCGC	CCGGAAGCTA	GGTGAGTGTG
80	90	100	110	120	130	140
GCATCCGAGC	TGAGGGAGCG	GAGCCTGAGA	CGCCGCTGCT	GCTCCGGCTG	AGTATCTAGC	TTGTCTCCCC
150	160	170	180	190	200	210
GATGGGATTTC	CCGTCCAAGC	TATCTCGAGC	CTGCAGCGCC	ACAGTCCCCG	GCCCTCGCCC	AGGTTCACTG
220	230	240	250	260	270	280
CAACCGTTCA	GAGTCCCCA	GGAGCTGCTG	CTGGCGAGCC	CGCTACTGCA	GGGACCTATG	GAGCCATTCC
290	300	310	320	330	340	350
GTAGTGCCAT	CCCGAGCAAC	GCACTGCTGC	AGCTTCCCCTG	AGCCTTTCCA	GCAAGTTTGT	TCAAGATTGG
360	370	380	390	400	(1)	
CTGTCAAGAA	TCATGGACTG	TTATTATATG	CCTTGTTTTC	TGTCAAGACA	CC ATG ATT CCT	
					MET Ile Pro	
417	432	447	462			
GGT AAC CGA	ATG CTG	ATG GTC	TTA TTA	TGC CAA	GTC CTG	CTA GGA
Gly Asn Arg	MET Leu	MET Val	Val Leu	Cys Leu	Gln Val	Leu Gly
						Ala

Figure 2/2

477	492	507
AGC CAT GCT AGT TTG ATA CCT GAG ACG GGG AAG AAA AAA GTC GCC GAG ATT CAG		
Ser His Ala Ser Leu Ile Pro Glu Thr Gly Lys Lys Val Ala Glu Ile Gln		
522	537	552
GGC CAC GCG GGA GGA CGC CGC TCA GGG CAG AGC CAT GAG CTC CTG CGG GAC TTC		
Gly His Ala Gly Gly Arg Arg Ser Gly Gln Ser His Glu Leu Leu Arg Asp Phe		
582	597	612
GAG GCG ACA CTT CTG CAG ATG TTT GGG CTG CGC CGC CCG CAG CCT AGC AAG		
Glu Ala Thr Leu Leu Gln MET Phe Gly Leu Arg Arg Arg Pro Gln Pro Ser Lys		
642	657	672
AGT GCC GTC ATT CCG GAC TAC ATG CCG GAT CTT TAC CGG CTT CAG TCT GGG GAG		
Ser Ala Val Ile Pro Asp Tyr MET Arg Asp Leu Tyr Arg Leu Gln Ser Gly Glu		
687	702	717
GAG GAG GAA GAG CAG ATC CAC AGC ACT GGT CTT GAG TAT CCT GAG CGC CCG GCC		
Glu Glu Glu Glu Gln Ile His Ser Thr Gly Leu Glu Tyr Pro Glu Arg Pro Ala		

Figure 2/3

747	762	777
AGC CGG GCC AAC ACC GTG AGG AGC TTC CAC CAC GAA GAA CAT CTG GAG AAC ATC		
Ser Arg Ala Asn Thr Val Arg Ser Phe His His Glu Glu His Leu Glu Asn Ile		
792	807	837
CCA GGG ACC AGT GAA AAC TCT GCT TTT CGT TTC TTC CTC TTT AAC CTC AGC AGC ATC	822	
Pro Gly Thr Ser Ser Glu Asn Ser Ala Phe Arg Phe Leu Phe Asn Leu Ser Ser Ile		
852	867	882
CCT GAG AAC GAG GTG ATC TCC TCT GCA GAG CTT CGG CTC TTC CGG GAG CAG GTG		897
Pro Glu Asn Glu Val Ile Ser Ser Ala Glu Leu Arg Leu Phe Arg Glu Gln Val		
912	927	942
GAC CAG GGC CCT GAT TGG GAA AGG GGC TTC CAC CGT ATA AAC ATT TAT GAG GTT		
Asp Gln Gly Pro Asp Trp Glu Arg Gly Phe His Arg Ile Asn Ile Tyr Glu Val		
957	972	987
ATG AAG CCC CCA GCA GAA GTG GTG CCT GGG CAC CTC ATC ACA CGA CTA CTG GAC		1002
MET Lys Pro Pro Ala Glu Val Val Pro Gly His Leu Ile Thr Arg Leu Leu Asp		

Figure 2/4

1017	1032	1047
ACG AGA CTG GTC CAC CAC AAT GTG ACA CGG TGG GAA ACT TTT GAT GTG AGC CCT		
Thr Arg Leu Val His His Asn Val Thr Arg Trp Glu Thr Phe Asp Val Ser Pro		
1062	1077	1092
CGG GTC CTT CGC TGG ACC CGG GAG AAG CAG CCA AAC TAT GGG CTA GCC ATT GAG		1107
Ala Val Leu Arg Trp Thr Arg Glu Lys Gln Pro Asn Tyr Gly Leu Ala Ile Glu		
1122	1137	1152
GTG ACT CAC CTC CAT CAG ACT CGG ACC CAC CAG GGC CAG CAT GTC AGG ATT AGC		1167
Val Thr His Leu His Gln Thr Arg Thr His Gln Gly Gln His Val Arg Ile Ser		
1182	1197	1212
CGA TCG TTA CCT CAA GGG AGT GGG AAT TGG GCC CAG CTC CGG CCC CTC CTG GTC		
Arg Ser Leu Pro Gln Gly Ser Gly Asn Trp Ala Gln Leu Arg Pro Leu Leu Val		
1227	1242	1257
ACC TTT GGC CAT GAT GGC CGG GGC CAT GCC TTG ACC CGA CGC CGG AGG GCC AAG		1272
Thr Phe Gly His Asp Gly Arg Gly His Ala Leu Thr Arg Arg Arg Ala Lys		
1287	1302	1317
CGT AGC CCT AAG CAT CAC TCA CAG CGG GCC AGG AAG AAG AAT AAG AAC TGC CGG		
Arg Ser Pro Lys His His Ser Gln Arg Ala Arg Lys Lys Asn Lys Asn Cys Arg		

Figure 2/5

1332(311)	1347	1362	1377
CGC CAC TCG CTC TAT GTG GAC TTC AGC GAT GTG GGC TGG AAT GAC TGG ATT GTG			
Arg His Ser Leu Tyr Val Asp Phe Ser Asp Val Gly Trp Asn Asp Trp Ile Val			
1392	1407	1422	1437
GCC CCA CCA GGC TAC CAG GCC TTC TAC TGC CAT GGG GAC TGC CCC TTT CCA CTG			
Ala Pro Pro Gly Tyr Gln Ala Phe Tyr Cys His Gly Asp Cys Pro Phe Pro Leu			
1452	1467	1482	
GCT GAC CAC CTC AAC TCA ACC AAC CAT GCC ATT GTG CAG ACC CTG GTC AAT TCT			
Ala Asp His Leu Asn Ser Thr Asn His Ala Ile Val Gln Thr Leu Val Asn Ser			
1497	1512	1527	1542
GTC AAT TCC AGT ATC CCC AAA GCC TGT TGT GTG CCC ACT GAA CTG AGT GCC ATC			
Val Asn Ser Ser Ile Pro Lys Ala Cys Cys Val Pro Thr Glu Leu Ser Ala Ile			
1557	1572	1587	
TCC ATG CTG TAC CTG GAT GAG TAT GAT AAG GTG GTA CTG AAA AAT TAT CAG GAG			
Ser MET Leu Tyr Leu Asp Glu Tyr Asp Lys Val Val Leu Lys <u>Asn Tyr Gln Glu</u>			

Figure 2/6

1602	1617	(408)	1636	1646	1656
ATG GTA GTA GAG GGA TGT GGG TGC CGC TGAGATCAGG CAGTCCCTTGA GGATAGACAG					
<u>MET Val Val Glu Gly Cys Gly Cys Arg</u>					
1666	1676	1686	1696	1706	1716
ATATACACAC CACACACACA CACCACATAC ACCACACACA CACGTTCCCA TCCACTCACC CACACACTAC					
1736	1746	1756	1766	1776	1786
ACAGACTGCT TCCTTATAGC TGGACTTTTA TTTAAAAAAA AATGGAAAAA ATCCCTAAAC					
1806	1816	1826	1836	1846	1856
ATTACCTTG ACCTTATTTA TGACTTTACG TGCAAAATGTT TTGACCATAT TGATCATATA TTTTGACAAA					
1876	1886	1896	1906	1916	1926
ATATATTTAT AACTACGTAT TAAAGAAGAAA AAATAAAATG AGTCATTATT TTAAAAAAA AAAAAAACT					
1946					
CTAGAGTCGA CGGAATTC					

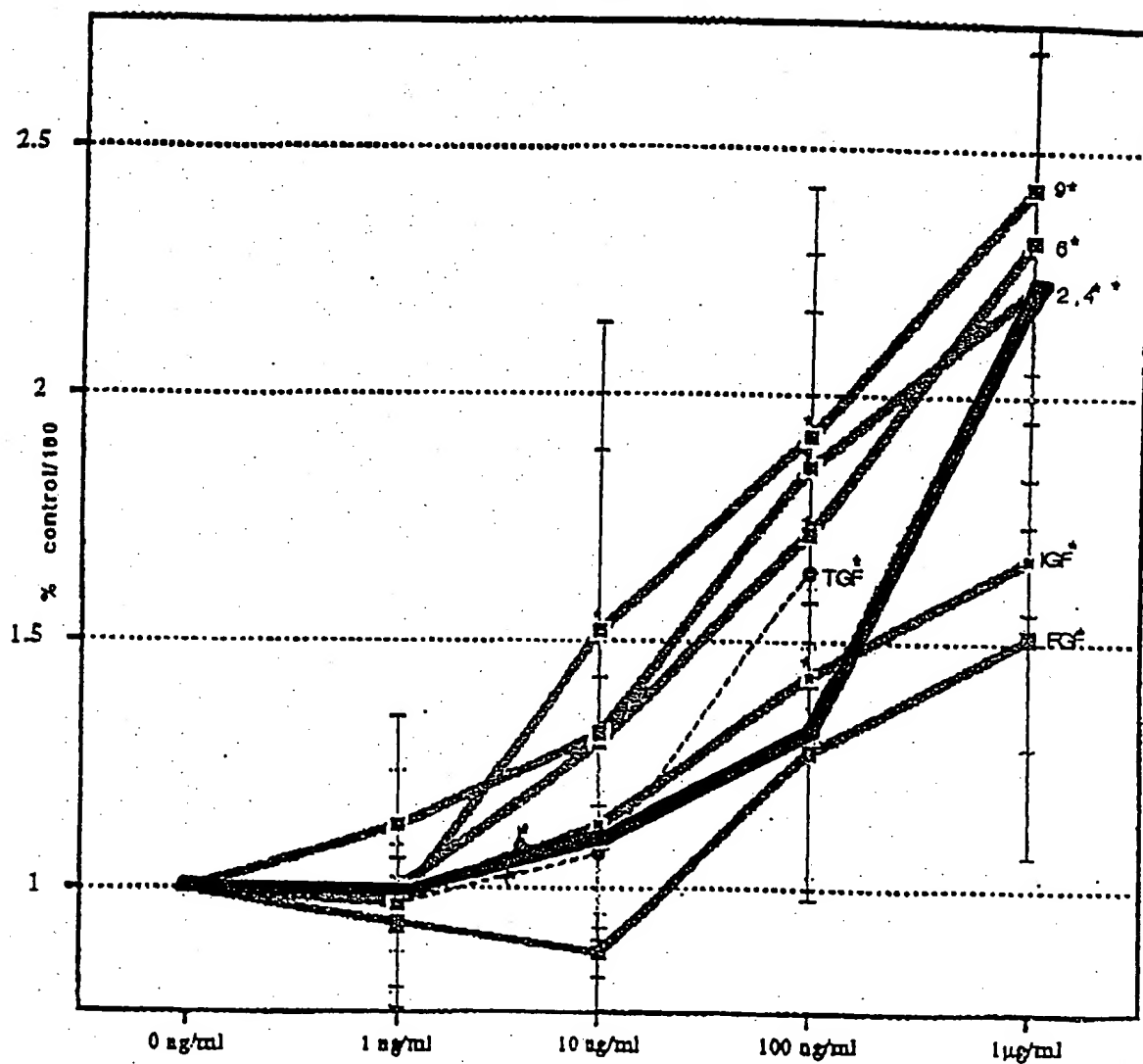
Figure 3

TGA ACA AGA GAG TGC TCA AGA AGC TGT CCA AGG ACG GCT CCA CAG AGG	48
* Thr Arg Glu Cys Ser Arg Ser Cys Pro Arg Thr Ala Pro Gln Arg	
-41 -40 -35	
CAG GTG AGA GCA GTC ACG AGG AGG ACA CGG ATG GCG CAC GTG GCT GCG	96
Gln Val Arg Ala Val Thr Arg Arg Thr Arg Met Ala His Val Ala Ala	
-25 -20 -15 -10	
GGG TCG ACT TTA GCC AGG CGG AAA AGG AGC GCC GGG GCT GGC AGC CAC	144
Gly Ser Thr Leu Ala Arg Arg Lys Arg Ser Ala Gly Ala Gly Ser His	
-5 1 5	
TGT CAA AAG ACC TCC CTG CGG GTA AAC TTC GAG GAC ATC GGC TGG GAC	192
Cys Gln Lys Thr Ser Leu Arg Val Asn Phe Glu Asp Ile Gly Trp Asp	
10 15 20	
AGC TGG ATC ATT GCA CCC AAG GAG TAT GAA GCC TAC GAG TGT AAG GGC	240
Ser Trp Ile Ile Ala Pro Lys Glu Tyr Glu Ala Tyr Glu Cys Lys Gly	
25 30 35	
GGC TGC TTC TTC CCC TTG GCT GAC GAT GTG ACG CCG ACG AAA CAC GCT	286
Gly Cys Phe Phe Pro Leu Ala Asp Asp Val Thr Pro Thr Lys His Ala	
40 45 50 55	
ATC GTG CAG ACC CTG GTG CAT CTC AAG TTC CCC ACA AAG GTG GGC AAG	336
Ile Val Gln Thr Leu Val His Leu Lys Phe Pro Thr Lys Val Gly Lys	
60 65 70	
GCC TGC TGT GTG CCC ACC AAA CTG AGC CCC ATC TCC GTC CTC TAC AAG	384
Ala Cys Cys Val Pro Thr Lys Leu Ser Pro Ile Ser Val Leu Tyr Lys	
75 80 85	
GAT GAC ATG GGG GTG CCC ACC CTC AAG TAC CAT TAC GAG GGC ATG AGC	432
Asp Asp Met Gly Val Pro Thr Leu Lys Tyr His Tyr Glu Gly Met Ser	
90 95 100	
GTG GCA GAG TGT GGG TGC AGG TAGTATCTGC CTGCGGG	470
Val Ala Glu Cys Gly Cys Arg	
105 110	

Figure 4

SULFATE INCORPORATION

Bovine Explants



n=5 BMPs

n=3 IGF-1, bFGF

n=1 TGFβ

* = different than control ($p < 0.05$)

BMP-2	■	BMP-4	—■—
BMP-6	—■—	BMP-9	—■—
IGF-1	—■—	bFGF	—■—
TGFβ	—●—		

Figure 5

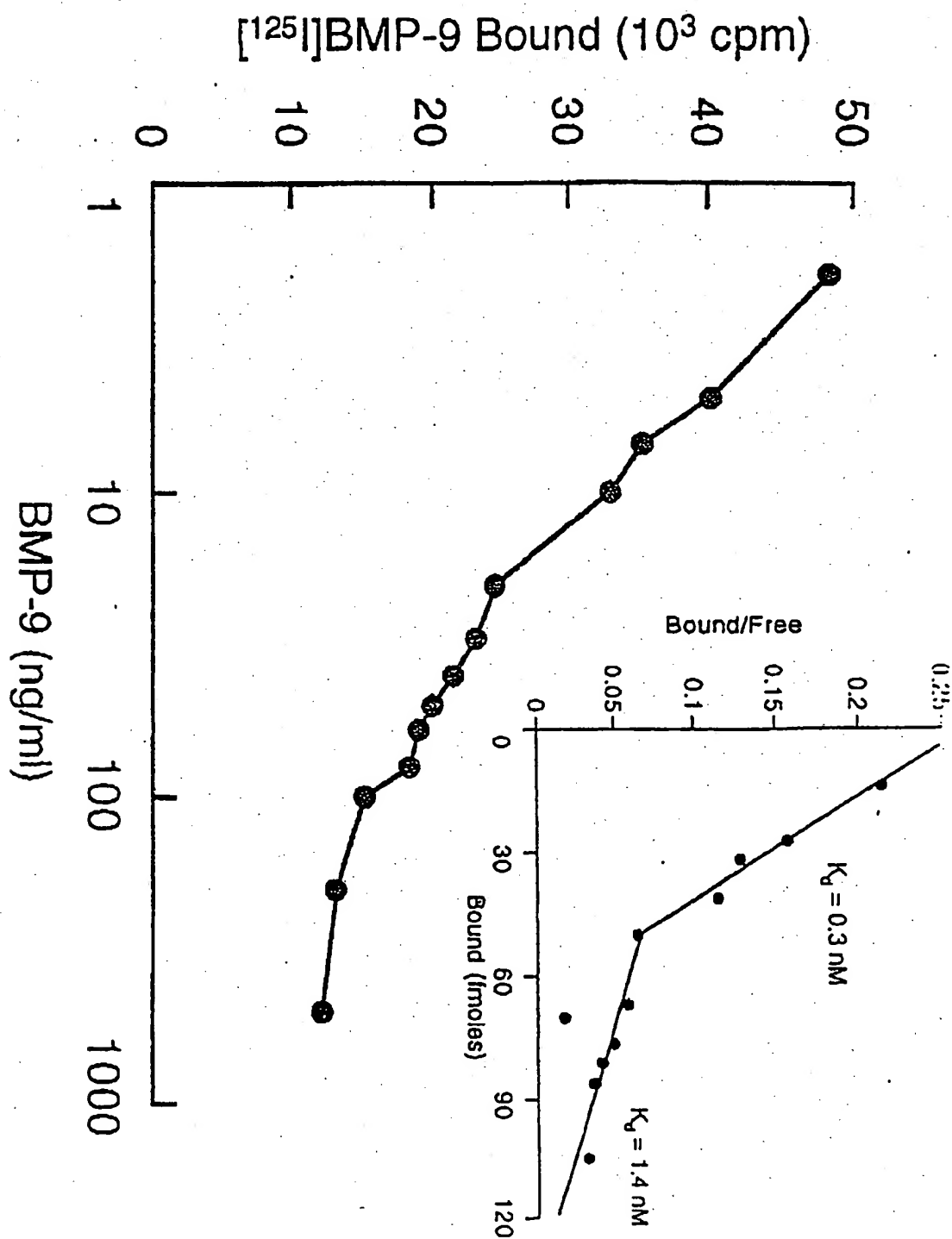


Figure 6/1

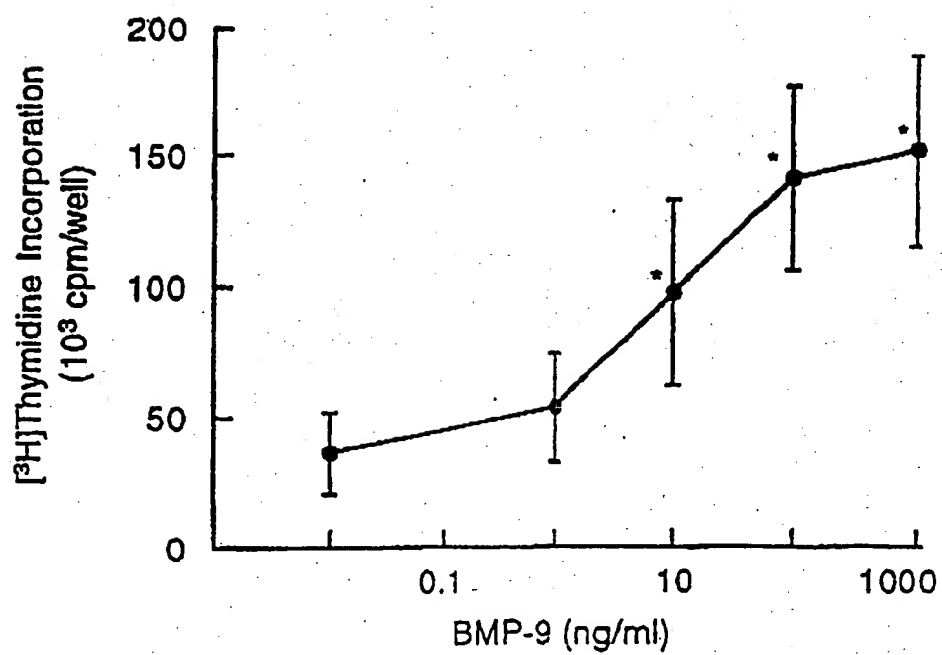


Figure 6/2

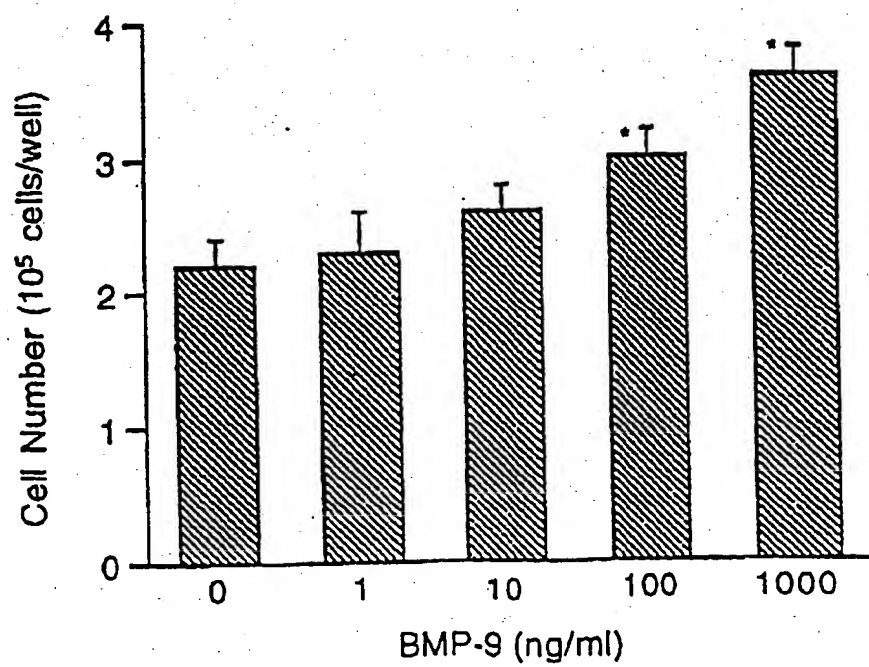
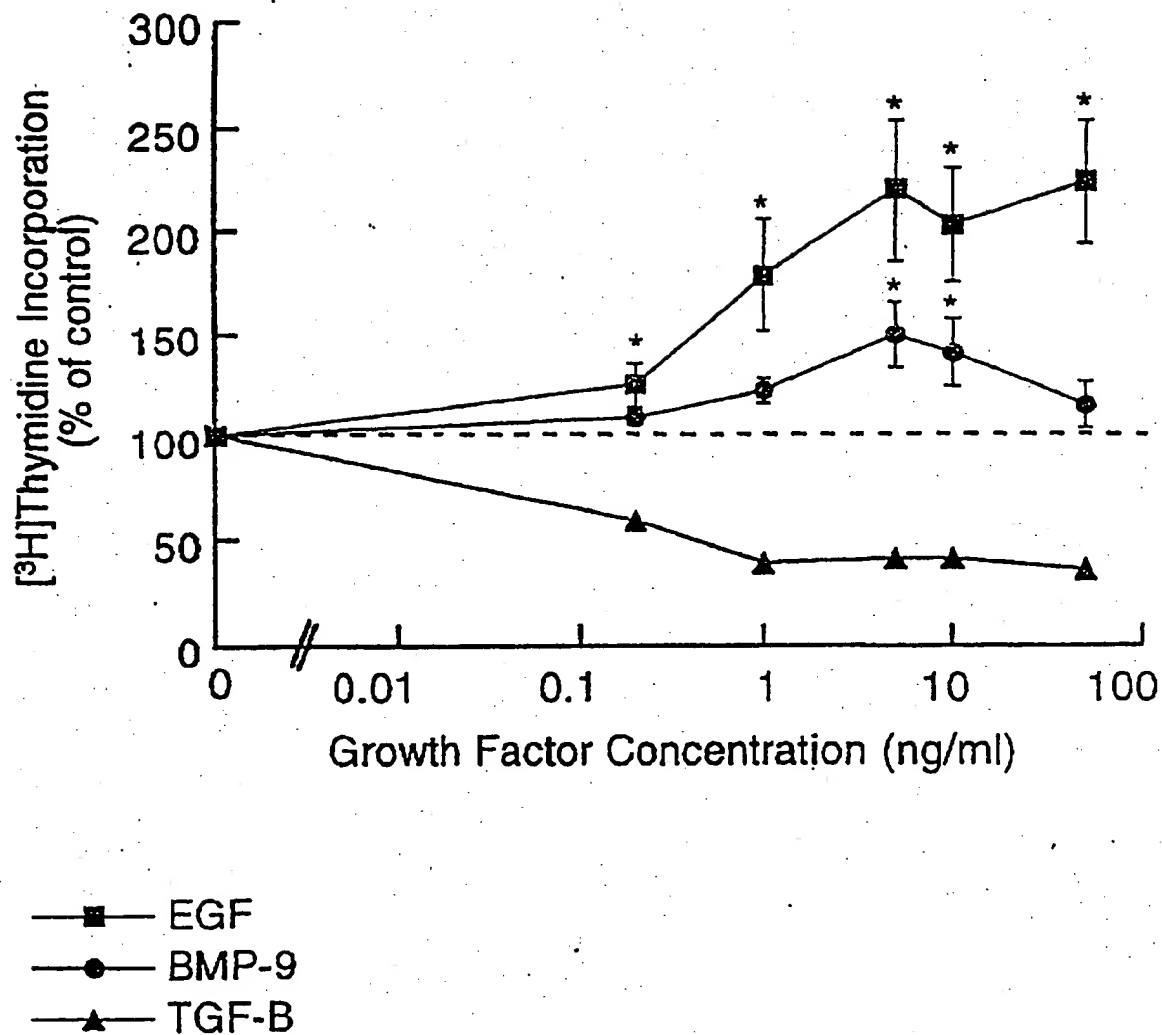


Figure 7



INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US 95/07084

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/51 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 00432 (GENETICS INST) 7 January 1993	7,8, 11-18,22
Y	see the whole document	20,21
Y	WO,A,94 06449 (CREATIVE BIOMOLECULES INC) 31 March 1994 see the whole document	20,21
	-/-	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- * "A" document defining the general state of the art which is not considered to be of particular relevance
- * "E" earlier document but published on or after the international filing date
- * "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- * "O" document referring to an oral disclosure, use, exhibition or other means
- * "P" document published prior to the international filing date but later than the priority date claimed

* "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

* "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

* "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

* "Z" document member of the same patent family

Date of the actual completion of the international search

18 October 1995

Date of mailing of the international search report

16.11.95

Name and mailing address of the ISA

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Authorized officer

Andres, S

INTERNATIONAL SEARCH REPORT

International Application
PCT/US 95/07084

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
O,P, X	<p>MOLECULAR BIOLOGY OF THE CELL, vol. 5, October 1994 page 384a SONG, J. ET AL. 'Bone morphogenetic protein-9 (BMP-9) binds to HEPG2 cells and stimulates proliferation' see abstract & 34th Ann.Meet. of the American Soc. for Cell Biol.; december 10-14, 1994; San Francisco, California</p> <p>-----</p>	20,21

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/07084

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 16,18,21 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☒ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claim 11 referring to a DNA sequence encoding BMP-8, has been interpreted as being meant to refer to BMP-9!
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US 95/07084

Patent document cited in search report	Publication date	Patent family member(s)	Publication date	
WO-A-9300432	07-01-93	AU-B-	652472	25-08-94
		AU-A-	2269992	25-01-93
		EP-A-	0592562	20-04-94
		JP-T-	6508990	13-10-94
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WO-A-9406449	31-03-94	AU-B-	4795193	03-03-94
		AU-B-	4797193	03-03-94
		AU-B-	4995593	03-03-94
		AU-B-	5129293	12-04-94
		AU-B-	5129393	12-04-94
		AU-B-	5162393	12-04-94
		AU-B-	5290893	12-04-94
		AU-B-	5590094	24-05-94
		CA-A-	2141554	17-02-94
		CA-A-	2141555	17-02-94
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		EP-A-	0661987	12-07-95
		EP-A-	0672064	20-09-95
		WO-A-	9403600	17-02-94
		WO-A-	9403075	17-02-94
		WO-A-	9403200	17-02-94
		WO-A-	9406447	31-03-94
		WO-A-	9406399	31-03-94
		WO-A-	9406420	31-03-94
		WO-A-	9410203	11-05-94